REGULATION OF SKELETAL MUSCLE INSULIN SENSITIVITY BY PAK1

Ragadeepthi Tunduguru

Submitted to the faculty of the University Graduate School
in partial fulfillment of the requirements
for the degree
Doctor of Philosophy
in the Department of Biochemistry and Molecular Biology
Indiana University

December 2016

Accepted by the Graduate Faculty, Indiana University, in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Debbie C. Thurmond, Ph.D., Co-chair

Doctoral Committee

_____
Jeffrey S. Elmendorf, Ph.D., Co-chair

September 6, 2016

_____
Simon J. Atkinson, Ph.D.

_____
Joseph T. Brozinick, Ph.D.

_____
Susan J. Gunst, Ph.D.

DEDICATION

This thesis is dedicated to my parents, Prasad and Shobha and to my husband, Halesha who have been a constant source of support and encouragement all through out my journey. I would also like to dedicate this thesis to my daughter, Spoorthi who is my little bundle of joy.

ACKNOWLEDGEMENTS

I would first like to thank my mentor Dr. Debbie Thurmond for her endless support, motivation and guidance through out my dissertation work. This life changing experience would not be possible without her careful guidance. She is an inspiring supervisor with excellent scientific and organizational skills and has been my role model. I am extremely fortunate to be supervised and mentored by Debbie who has provided me with ample research opportunities to develop my scientific credentials and intellectual freedom that has enabled me to successfully complete my dissertation.

I would also like to thank all my committee members Dr. Jeffrey Elmendorf, Dr. Simon Atkinson, Dr. Susan Gunst and Dr. Joseph Brozinick for their valuable inputs and continuous guidance in shaping and molding my thesis project into a fruitful product. I would like to thank Dr. Elmendorf for his remarkable generosity during the last year of my PhD in providing lab space to continue my research work and also for his valuable advice for crucial experiments that benefited my project greatly. I would also like to thank Dr. Brozinick for providing kind support and expertise in performing some of the key experiments that were critical for my project.

I would like to thank all the current and former members of the Thurmond lab: Eunjin Oh, Miwon Ahn, Rajakrishnan Veluthakal, Jing Zhang, Zhanxiang Wang, Stephanie Yoder, Mike Kalwat, Latha Ramalingam, Arianne Aslamy, Karla Elzein, Vishal Salunkhe, April Hoggatt and Erika Olson for all the cheerful and engaging conversations in the lab and for supporting me through out my graduate work. I

cherish all the funny moments and scientific conversations with Latha and Stephanie during my initial days in the Thurmond lab. I would like to thank Latha for being such a great friend who has helped and supported me both professionally as well as personally and for being there all the times whenever I needed a friend. I would like to thank Arianne for all the wonderful moments spent together in and outside the lab in the past two years. I would like to thank Raja for all the scientific conversations and career advice that helped me greatly. Thanks to Arianne and Karla for making me feel very special on my most important days of this journey and for all the wonderful memories in the lab.

My stay in US and specifically in Indianapolis would not have been a wonderful experience without these great friends who have become family in the last few years. I would like to thank Abhi, Sridhar, Rakesh, Minal, Vinay, Lakshmi, Sidharth, Isha, Akaash, Kishore, Aarthi, Chandra, Reddy and Dharunesh for all the happier times spent together playing pranks on one another and enjoying some awesome food.

Finally, I would like to thank my brother Deepak and sister Jyostna for their unceasing love and encouragement. This wonderful journey would not have been possible without the immense support of my parents, Prasad and Shobha and my husband, Halesha who believed in me and wanted the best for me.

Ragadeepthi Tunduguru

Regulation of skeletal muscle insulin sensitivity by PAK1

Insulin-stimulated glucose uptake into skeletal muscle cells requires translocation of the glucose transporter-4 (GLUT4) from the cell interior to the plasma membrane. Insulin-stimulated GLUT4 vesicle translocation is dysregulated in Type 2 diabetes (T2D). The Group I p21–activated kinase (PAK1) is a required element in insulin-stimulated GLUT4 vesicle translocation in mouse skeletal muscle *in vivo*, although its placement and function(s) in the canonical insulin signaling cascade in skeletal muscle cells, remain undetermined. Therefore, the objective of my project is to determine the molecular mechanism(s) underlying the requirement for PAK1 in the process of insulin-stimulated GLUT4 vesicle translocation and subsequent glucose uptake by skeletal muscle cells.

Toward this, my studies demonstrate that the pharmacological inhibition of PAK1 activation blunts insulin-stimulated GLUT4 translocation and subsequent glucose uptake into L6-GLUT4myc skeletal myotubes. Inhibition of PAK1 activation also ablates insulin-stimulated F-actin cytoskeletal remodeling, a process known to be required for mobilizing GLUT4 vesicles to the plasma membrane. Consistent with this mechanism, PAK1 activation was also required for the activation of cofilin, another protein implicated in F-actin remodeling. Interestingly, my studies reveal a novel molecular mechanism involving PAK1 signaling to p41-ARC, a regulatory subunit of the cytoskeletal Arp2/3 complex, and its interactions with another cytoskeletal factor, N-WASP, to elicit the insulin-stimulated F-actin remodeling in skeletal muscle cells. Pharmacological inactivation of N-WASP fully abrogated

insulin-stimulated GLUT4 vesicle translocation to the cell surface, coordinate with blunted F-actin remodeling.

Furthermore, my studies revealed new insulin-induced interactions amongst N-WASP, actin, p41-ARC and PAK1; inactivation of PAK1 signaling blocked these dynamic interactions. Taken together, the above studies demonstrate the significance of PAK1 and its downstream signaling to F-actin remodeling in insulin-stimulated GLUT4 vesicle translocation and glucose uptake, revealing new signaling elements that may prove to be promising targets for future therapeutic design.

Debbie C. Thurmond, Ph.D., Co-chair

Jeffrey S. Elmendorf, Ph.D., Co-chair

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LIST OF ABBREVIATIONS

ADP	Adenosine diphosphate
ANOVA	Analysis of variance
Arp2/3	Actin-related protein 2/3 complex
p41-ARC	Actin-related protein 2/3 complex subunit 1A
AS160	Akt substrate of 160 kDa
BSA	Bovine serum albumin
CCL2	C-C motif chemokine ligand 2
Cdc42	Cell division cycle 42
DAG	Diacyl glycerol
ECM	Extracellular matrix
ECL	Enhanced chemiluminescence
ER	Endoplasmic reticulum
FBS	Fetal bovine serum
GLUT	Glucose transporter
GTP	Guanosine Triphosphate
GTPase	Guanosine Triphosphatase
GSV	GLUT4 storage vesicle
GWAS	Genome-wide association study
IB	Immunoblot
IGF	Insulin-like growth factor
IgG	Immunoglobulin G
IL6	Interleukin 6

IP	Immunoprecipitation
IPA3	Inhibitor of PAK activation
IRS	Insulin receptor substrate
JNK	Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MODY	Maturity onset diabetes of the youth
NF-κB	Nuclear factor κB
NP-40	Non-ionic P-40 detergent
N-WASP	Neuronal Wiskott-Aldrich syndrome protein
PAGE	Polyacrylamide gel electrophoresis
PAK1	p21 activated kinase 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
PI3K	Phosphotidylinositol-3-kinase
PIP	Phosphatidylinositol 4-phosphate
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKB	Protein kinase B
PKC	Protein kinase C
PM	Plasma membrane
PMSF	Phenylmethylsulfonylfluoride
PVDF	Polyvinylidine difluoride
Rac1	Ras-related C3 botulinum toxin substrate 1

Rho	Ras homologous
SDS	Sodium dodecyl sulfate
Ser	Serine
SNAP	Synaptosome associated protein
SNARE	Soluble N-ethylmaleimide sensitive factor attachment protein receptor
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TNF-α	Tumor necrosis factor α
Thr	Threonine
Tyr	Tyrosine
VAMP-2	Vesicle associated membrane protein-2

CHAPTER 1

INTRODUCTION

1.1. OVERVIEW OF DIABETES

Glucose is the principal cellular energy source in humans. Hence maintenance of glucose homeostasis is critical for survival. There is an elaborate network of regulatory mechanisms, involving multiple organs and endocrine systems, developed to control the levels of glucose in the body. After the consumption of food, there is an increase in glucose levels in the blood due to digestion. This increase in blood glucose level triggers the pancreatic β cells to secrete the hormone insulin into the blood stream. Insulin then communicates with peripheral tissues of the body (such as skeletal muscle and adipose tissues) to increase the absorption of excess glucose, and with the liver to limit its production of glucose, culminating in the restoration of resting level of blood glucose. Conversely, during fasting conditions, the decrease in blood glucose levels signals pancreatic α cells to secrete the counter-regulatory hormone glucagon, which then enhances the production and secretion of glucose into the blood by the liver. These coordinated actions of insulin and glucagon maintain glucose homeostasis and euglycemia. Any abnormalities in these regulatory mechanisms lead to breakdown of glucose homeostasis, prompting metabolic abnormalities that can precipitate the development of 'Diabetes'.

Diabetes is a complex metabolic disorder with multiple etiologies resulting in dysregulation of glucose homeostasis wherein glucose levels are higher than the normal range (hyperglycemia). The normal range of blood glucose levels in healthy individuals are 70 – 100 mg/dL during the fasting state, and less than 140 mg/dL during post-prandial state [1]. According to the Center for Disease Control

(CDC), as of 2012 there are 29.1 million diabetes patients in the United States, and additional 86 million Americans with pre-diabetic conditions. The American Diabetes Association classifies an individual as prediabetic or diabetic based on the fasting blood glucose levels. In prediabetic patients the fasting blood glucose levels are in the range of 100 – 125 mg/dl and in diabetic patients the levels are above 125 mg/dL. There are four types of diabetes:

a) **Type 1** (T1D, also called Juvenile diabetes) – Type 1 diabetic patients make up ~5-10% of adult diabetic patients. This is an autoimmune disorder wherein insulin secreting β-cells are destroyed by the immune system resulting in decreased insulin production and thus increased blood glucose levels.

b) **Type 2** (T2D) – Type2diabetes (also known as non-insulin dependent diabetes mellitus) is a chronic metabolic disorder characterized by high blood glucose levels (hyperglycemia) due to three key defects: 1) increased hepatic glucose production, 2) failure to secrete sufficient insulin and 3) impaired insulin action in liver and peripheral tissues such as skeletal muscle and adipose tissues (also termed as insulin resistance). Insulin resistance is discussed in detail in section 1.2. Approximately 90% of adult diabetic patients have type 2 diabetes and it has been well established that obesity and physical inactivity are the two major risk factors for the development of this disorder. Dysregulation of metabolism in T2D which is also known as a multiorgan disease affects all the other organs/ tissues that result in secondary complications including

hypertension, non-alcoholic fatty liver disease and metabolic syndrome all of which are associated with increased cardiovascular problems.

c) **Gestational diabetes** – According to the CDC, the prevalence of gestational diabetes is around 4-9% of pregnancies. This occurs in women around the 24th week of gestation. Gestational diabetes is characterized by insulin resistance in pregnant women, leading to increased blood glucose levels. Nearly 5-10% of women develop T2D just after pregnancy, and around 8-13 % will have ~60% chance of developing T2D later in their life.

d) **Maturity-onset diabetes of young (MODY)** – This accounts for 1-2% of the diabetic patient population. MODY is caused by genetically heritable mutations. These mutations are monogenic and are autosomal dominant. The most common genetic mutations in MODY occurs in glucokinase (GCK) and hepatocyte nuclear factor (HNF) 1A/4A genes. Mutations in GCK cause mild and stable fasting hyperglycemia. Mutations in HNF1A and HNF4A cause progressive pancreatic β-cell dysfunction.

1.2. INSULIN RESISTANCE

In a healthy individual, the pancreatic β-cells synthesize insulin constantly irrespective of the blood glucose levels. The synthesized insulin is stored in granules, and once the β-cells are triggered by elevation of blood glucose levels, the granules fuse with the plasma membrane and insulin cargo is released into the bloodstream. In the bloodstream, insulin then travels to and impacts various

insulin-responsive cell types of the body. Binding to the insulin receptor at the cell surfaces of skeletal muscle cells and adipocytes, insulin evokes an increase in the uptake of glucose into these cells, thereby decreasing the blood glucose levels. However, when insulin no longer is able to elicit its blood glucose-lowering effects, condition called 'insulin resistance' ensues [2]. In this condition, the body produces insulin, but cannot respond to normal levels of the insulin to maintain normal blood glucose levels. As a result, hyperglycemia occurs and in order to compensate for this, β cells increase insulin production by increasing their mass to be able to release much higher than normal levels of insulin into the bloodstream. In time this stress to overproduce insulin results in β cell dysfunction and the beta cell can no longer cope with the hyperglycemia, leading to destruction of the β cells. This results in decreased insulin production, and the development of frank T2D. Thus, insulin resistance is a pre-stage before T2D.

Insulin resistance is considered to be a post-insulin receptor disorder, wherein the problem lies with the cells that respond to insulin rather than cells that produce insulin. Though several mechanisms have been proposed to explain the pathogenesis/etiology of insulin resistance, three mechanisms are widely accepted: ectopic lipid accumulation, development of endoplasmic reticulum (ER) stress and systemic inflammation [3].

1.2.1. Ectopic lipid accumulation

There is a strong connection between obesity and T2D [4]. Continued exposure of tissues to nutrient overload results in accumulation of toxic byproducts in

insulin responsive cells, especially lipid-derived metabolites that accumulate in liver, skeletal muscle and heart in response to high-fat diets. The current theory in the field is that high-fat diet induces accumulation of lipid species in cells due to impairment of normal fatty acid oxidation. As a result diacylglycerols (DAGs), ceramides and triglycerides are accumulated in cells [3, 5-7]. DAGs, being intracellular signaling molecules, activate Protein kinase C (in skeletal muscle, PKCθ), that in turn blocks activation of insulin receptors. Similarly, ceramide impairs activation of V-AKT Murine Thymoma Viral Oncogene Homolog 2(AKT2) by activating protein phosphatase 2A, which dephosphorylates AKT2. Thus, insulin signaling is inhibited due to accumulation of lipid species due to incomplete oxidation of fatty acids [7].

1.2.2. Development of ER stress

In response to excess nutritional exposure such as high fat diets, insulin secreting tissues such as pancreatic β cells and insulin sensitive tissues such as adipose tissue and liver develop ER stress, which in turn activates the unfolded protein response (UPR) [8]. New reports suggest that the UPR negatively regulates insulin signaling and also increases lipid accumulation in liver and adipose tissue [9], which in turn causes insulin resistance.

1.2.3. Systemic inflammation

The innate immune system is known to play a major role in insulin resistance in obese patients. The expression of Tumor necrosis factor (TNF)-α is increased in adipose tissues of obese subjects, and this increased TNF-α derived from

activated macrophages located within the adipose tissue (adipose tissue macrophages, ATMs) [10-12]. These ATMs are recruited by the chemokines Monocyte chemoattractant protein-1(MCP1) or C-C Motif chemokine ligand-2 (CCL2) that are expressed by adipocytes present in the adipose tissue matrix. The increased TNF-α released from the ATMs stimulates NF-κB signaling in the adipocytes, leading to the increased production and release of cytokines, such as IL-1 and IL-6 by the adipose tissue. IL-6 then increases adipocyte lipolysis and blocks insulin signaling by promoting degradation of the insulin receptor substrates 1 and 2 (IRS1 and IRS2), thereby decreasing binding of IRS1 and IRS2 to insulin receptors, impeding the progression of the insulin signaling cascade [3].

It is now clear that metabolic aberrations, ER stress and inflammatory signaling are all associated and influence each other. Deciphering these mechanisms at the molecular level is crucial to understand the insulin signaling mechanisms under pathological conditions in order to develop new therapeutic strategies to combat progression to T2D.

1.3. GLUCOSE TRANSPORTERS

Whole body glucose homeostasis is maintained by three critical steps: 1) postprandial glucose absorption into the small intestine, 2) synthesis and production of glucose by the liver, and 3) glucose uptake by all the tissues. Glucose uptake /transport of glucose across cellular membranes in mammalian cells occurs via two major types of transporters, namely sodium-dependent glucose transporters (SGLTs) and glucose transporters (GLUTs).

1.3.1. Sodium-dependent glucose transporters (SGLT)

This transporter family consists of six isoforms, SGLT1-SGLT6 [13-15], each of which co-transports glucose along with sodium ions. In the intestine, SGLTs facilitate transport across the apical membrane of the intestinal epithelium; this is driven by the concentration gradient of each on either side of the membrane[16]. This concentration gradient is maintained by the coordinated actions of two facets to the SGLT co-transporters: the energy-driven sodium/potassium ATPase, which transports sodium ions, and a glucose transporter that transports glucose across the basolateral membrane into the circulation.

SGLT2 inhibitors such as Canagliflozin [17, 18] [19](Trade name-Invokana) and Dapagliflozin [20] (Trade name-Farxiga) are the newly developed oral anti-diabetic drugs on the market, and are also available in combination with metformin (first line medication for treatment of T2D). SGLT2 inhibitors have a unique mechanism of action, in which they inhibit re-absorption of glucose in the proximal convoluted tubules (PCT) of kidney to facilitate its excretion in urine [21, 22]. This action is dependent on blood glucose levels but not on the action of insulin. However, these medications carry a risk of side effects, including diabetic ketoacidosis, acute kidney injury and cardiovascular complications [23]. Nevertheless, their unique mechanism of action and positive pleotropic effects to reduce body weight and blood pressure make them attractive options as add-on therapies for T2D patients not well-controlled by other medications [24].

1.3.2. Glucose transporters (GLUTs)

As opposed to the passive glucose transport of the SGLTs, GLUTs mediate the facilitative transport of glucose. GLUTs comprise 14 family members (GLUT1-GLUT14) located in a variety of tissues. Each GLUT isoform contains the characteristic 12 transmembrane helices, and a conserved N-glycosylation consensus site (Asn-X-Ser/Thr) typically positioned in the first or fifth exofacial loop between the transmembrane helices [25]. Based on primary sequence comparisons, GLUT family proteins are divided into three sub-classes: Class I, II and III facilitative transporters [26].

a) Class I facilitative transporters – GLUT1-4 and -14 belong to the Class I transporters. GLUT1 is the most extensively studied and is ubiquitously expressed. With highest expression levels in erythrocytes and endothelial cells of the brain [27], GLUT1 catalyzes the rate limiting step in supplying glucose to the cells of central nervous system. Supporting this, studies by Seidner et al have linked GLUT1 deficiency in humans to defective transport of glucose across the blood brain barrier [28]. GLUT2 is the low affinity yet high capacity glucose transporter with predominant expression in pancreas, liver, kidney and basolateral membranes of the small intestine. GLUT2 transports the majority of the glucose into the liver, and plays a vital role in glucose-stimulated insulin secretion from pancreatic β cells [29, 30]. In a study by E Brot-Laroche et al (2003) using pre-fasted mice, the recruitment of GLUT2 to the apical membrane of the intestinal epithelium was demonstrated to occur within minutes in

response to a glucose bolus and the elevation of circulating glucose concentration [31]. GLUT2 has the highest Km (~17 mM) for glucose amongst the 14 GLUT family members. GLUT2 also transports galactose, fructose, and mannose; GLUT2 transports glucosamine with even higher affinity [32]. GLUT3 is high-affinity glucose transporter (Km=1.4 mM), and is predominantly expressed in tissues with high glucose demand, including brain, testes and placenta. Additionally, GLUT3 is present in platelets, lymphocytes and neutrophils. In lymphocytes GLUT3 was localized to vesicles that underwent stimulus-induced translocation from an intracellular locale to the plasma membrane [33]. In a process analogous to this, GLUT4 was also found to translocate to the cell surface of insulin responsive cell types such as skeletal muscle, adipose tissue and cardiac muscle [34]. Since its discovery in 1989, GLUT4 translocation has been noted as the rate-limiting step of insulin-stimulated glucose uptake into peripheral tissues. More details about GLUT4 and its regulation are in section 1.3.3. GLUT14, which is 95% identical to GLUT3, is exclusively expressed in testis and is suggested to be a product of gene duplication of GLUT3 [35]. With no orthologue in mice [36], GLUT14 has no known role in glucose metabolism in expressed tissue.

b) Class II facilitative transporters – The class II family of GLUTs includes GLUT5, GLUT7, GLUT9 and GLUT11. This classification is based on the substrate specificity of each for fructose. Amongst these, GLUT5 is predominantly expressed in small intestine, with lower levels of

expression in kidney, brain and testis. GLUT5 is the major fructose transporter apart from GLUT2, yet has only minimal activity in glucose transport. Moreover, growing evidence suggests a correlation between GLUT5 expression levels and fructose uptake rates, with that of metabolic disturbances underlying conditions such as obesity, hypertension and diabetes. GLUT7 has ~74% amino acid sequence similarity to that of GLUT5 [37], and is expressed primarily in the apical membrane of small intestine and colon [38]. GLUT7 is the most recently characterized GLUT, found to have high affinity for glucose and fructose (Km for glucose and fructose = 0.3mM) [38]. However, because the distribution of GLUT7 in small intestine (primarily expressed) does not entirely match with the availability of glucose and fructose [37], these are not considered to be the physiological substrates for GLUT7. GLUT9, initially discovered as a high-affinity glucose (Km=0.6 mM) / fructose (Km=0.4 mM) transporter, has also been identified as a high-affinity uric acid transporter (Km=0.9 mM) that plays a major role in urate homeostasis [39]. GLUT11, another fructose transporter, has low affinity for glucose (Km=0.16 mM) [40, 41] and is predominantly expressed in pancreas, kidney and placenta, with moderate expression levels in heart and skeletal muscle. Unlike GLUT4, GLUT11 is exclusively expressed in slow twitch muscle fibers, with no changes reported to its expression levels under pathophysiological conditions [42].

c) Class III facilitative glucose transporters – Though classified third of the three classes of GLUTs, the class III GLUTs are suggested to have evolved before the class I and II transporters, having the highest homologies with different transporters in yeast, bacteria and *Drosophila melanogaster* [43]. There are structural features common to all of the class III transporters: an N-glycosylation site at the fifth extracellular loop, and an internalization signal (dileucine) that is required for the intracellular localization under steady-state conditions. The low affinity glucose transporter, GLUT6, is predominantly expressed in brain, spleen and leukocytes, and has been suggested to have no role in insulin-stimulated glucose uptake. Unlike GLUT6, GLUT8 has a high affinity for glucose (Km~2 mM) [44] and GLUT8's activity is specifically inhibited by D-fructose and D-Galactose, suggesting it to be a multifunctional transporter [44]. Predominantly expressed in testes, GLUT8 has also been detected in insulin-sensitive tissues such as heart and skeletal muscle. Interestingly, recent studies using GLUT8 deficient mice fed a high-fructose diet revealed the potential role of GLUT8 to be in mediating deleterious effects of fructose in inducing glucose intolerance and dyslipidemia [45]. These data suggest GLUT8 to have potential as a novel therapeutic target to treat or possibly prevent obesity and metabolic syndrome. GLUT10 is predominantly expressed in liver and pancreas, and while GLUT10 is localized within the T2D-linked region on human chromosome 20q12-13.1, GLUT10 polymorphisms were not associated with T2D in humans [46-48].

GLUT12 is largely expressed in insulin-responsive tissues such as pancreas, skeletal muscle and adipose tissue, and is considered as the secondary insulin-sensitive glucose transporter behind GLUT4, due to its translocation to the plasma membrane along with GLUT4 in response to insulin in skeletal muscle [49]. Finally, GLUT13, also called H+/-myo-inositol transporter (HMIT), is predominantly expressed in brain and contains all of the motifs required for glucose transport activity. Surprisingly however, GLUT13 transports myo-inositol with a Km of 100 μM [50] and lacks sugar transport activity.

1.3.3. GLUT4 is the major insulin-responsive glucose transporter

The major route of clearance of postprandial serum glucose is via the skeletal muscle. In skeletal muscle, glucose is oxidized to meet the energy demands of the cell, and glucose is also stored as energy in the form of glycogen following glucose transport. Of the 13 currently identified sugar transporter proteins known, the principal glucose transporter that mediates glucose clearance and uptake into skeletal muscle to regulate whole body glucose homeostasis, is GLUT4. While skeletal muscle also contains GLUT1, GLUT5 and GLUT12, which contribute significantly to the glucose uptake, GLUT4 is the only insulin-responsive GLUT that redistributes from intracellular regions to the plasma membrane in response to insulin. The GLUT4 protein contains unique phenylalanine residues in the N-terminal sequence [51-55], as well as dileucine and acidic motifs in the C-terminal region [56-60], which are thought to facilitate its membrane trafficking

capability. Moreover, both the amino- and carboxy-termini of GLUT4 are located in the cell interior/cytoplasm.

Early evidence for the significance of GLUT4 in the maintenance of whole-body glucose homeostasis comes from studies using classic, whole-body heterozygous GLUT4+/- gene ablated mice, with reduced GLUT4 expression both in skeletal muscle and adipose tissue [61, 62]. As consistent with the prior ex vivo work on GLUT4, the heterozygous GLUT4+/- mice exhibited insulin resistance and predisposition toward diabetes. In contradiction however, the whole-body *homozygous* $GLUT4^{-/-}$ knockout mice demonstrated no major metabolic defects [63]; studies suggested this was due to possible upregulation of compensatory mechanisms. Later, transgenic mice engineered to overexpress GLUT4 in adipose or skeletal muscle tissues were highly insulin-sensitive and glucose tolerant [64-66], supportive of the important physiological reliance upon GLUT4 in these tissues for the overall regulation of whole-body glucose homeostasis.

1.3.4. Endogenous expression and tissue distribution of GLUT4

Maintenance of glucose homeostasis requires the concerted action of different insulin-secreting tissues as well as insulin-sensitive tissues - any defect in one or more tissue types results in T2D. Insulin-sensitive tissues act by mobilizing the GLUT4 containing vesicles from intracellular pools to the plasma membrane (PM) in response to insulin [67-69], and this increases the glucose uptake by 10-20 fold. While GLUT4 is widely distributed in insulin-responsive tissues including skeletal muscle, brown adipose tissue (BAT), white adipose tissue (WAT) [70,

71] and heart, it is also in brain and has been proposed to translocate in response to insulin to contribute to whole-body glucose homeostasis in sync with the other GLUT4-expressing tissues. Studies of tissue–specific/conditional GLUT4 knockout mouse models have shed light upon the relative contribution in each tissue in isolation to overall glucose homeostasis [72]. For example, mice deficient in GLUT4 specifically in skeletal muscle exhibited severe insulin resistance and glucose intolerance at an early age [73]. Similarly, adipose-selective depletion of GLUT4 in mice led to impaired glucose tolerance and hyperinsulinemia, indicating a critical role of adipose tissue in glucose homeostasis [74]. The data showing importance of GLUT4 expression and function in skeletal muscle and adipose tissue translate to human findings as well. In particular, decreased GLUT4 levels in adipose tissue correlate with increased obesity in humans, and conversely, increased GLUT4 expression is seen in exercised individuals [75-78]. Towards this, a 2.4 kb DNA segment at the 5' region of human GLUT4 gene has been identified as the regulatory element for the tissue specific expression of GLUT4 (at the transcription level) in adipose, skeletal muscle and cardiac muscle tissues [70, 79]. Discovering new regulatory elements for tissue specific expression in metabolic tissues that regulate circulating glucose levels will provide new avenues and strategies to treat this debilitating disease.

1.4. GLUCOSE UPTAKE INTO SKELETAL MUSCLE

1.4.1. Insulin-independent/ Contraction-stimulated glucose uptake in skeletal muscle

In brief, contraction and exercise-induced glucose uptake is regulated at three different levels- 1) Skeletal muscle GLUT4 expression, 2) GLUT4 mobilization to the sarcolemma and T-tubules and 3) Glucose transport/ uptake into skeletal muscle.

1.4.1.1. Skeletal muscle GLUT4 expression:

Evidence for the regulation of skeletal muscle GLUT4 expression impacting glucose homeostasis stems from early studies in 1996 [65], where transgenic mice overexpressing GLUT4 in skeletal muscle showed improved glucose tolerance and, enhanced glucose disposal. Since then, much focus has been shifted towards identifying therapeutic strategies to increase the expression of GLUT4 as a means to address metabolic disorders including insulin resistance, metabolic syndrome/prediabetes and T2D. The levels of GLUT4 expression differ in different skeletal muscle fiber types, with higher expression levels of GLUT4 in the type I oxidative fibers (soleus muscle) compared to the glycolytic type II fibers (epitrichlearis and extensor digitorum longus (EDL)) [80-82]. Longer duration exercise is implicated in the conversion of type II to type I muscle fibers [83], increasing the efficiency of the muscle in utilizing glucose; i.e. type I muscle fibers produce more ATP (being oxidative goes through mitochondria) than glycolytic (anerobic glycolysis) which produces less ATP per glucose molecule. For example, in as few as 3 hours post-swimming, including a 45 min rest period, an increase in GLUT4 mRNA expression associated with a surge in GLUT4 protein expression is observed [84, 85].

Two key domains in the GLUT4 promoter region have been identified that regulate the GLUT4 gene expression specifically in adipose tissue, skeletal muscle and cardiac muscle. The first region, Domain 1, is activated by the binding of glucose enhancer factor (GEF) and members of the myocyte enhancer factor 2 (MEF2) family of transcription factors, MEF2A and MEF2D. The second region, the MEF2 domain, contains an apparent MEF2 binding region, and has been suggested to confer tissue specific expression [86]. Conversely, the transcriptional repression of MEF2 is carried out by HDAC5, a class II histone deacetylase (HDAC). Specifically, GEF and MEF2 were discovered as the regulatory elements that control the tissue specific expression of GLUT4 gene in skeletal muscle [87, 88]. GEF and MEF2 activities are initiated by a surge in AMP/ATP ratio and intracellular calcium concentrations during exercise. This in turn induces the activation of the energy sensor of the cell, 5'-AMP activated protein kinase (AMPK) and Ca^{2+}/calmodulin-dependent protein kinase (CAMK) II, respectively. Activated AMPK and CAMK II increase GEF and MEF2A DNA binding [89-91], as well as to remove the HDAC5 repression (by increasing its nuclear export), to ultimately increase GLUT4 mRNA expression.

1.4.1.2. GLUT4 mobilization to sarcolemma and T-tubules:

The number of GLUT4 proteins integrated into the cell surface/PM depends on the rate of endocytosis and exocytosis of GLUT4-containing vesicles in response to various stimuli. Although insulin is known to regulate this mobilization process, primarily by increasing the GLUT4 vesicle exocytosis, exercise/ contraction both increases GLUT4 exocytosis and decreases GLUT4 endocytosis. Studies by Klip

et al demonstrated that acute insulin injection into rat hindlimb muscle (*in vivo*) resulted in a surge of GLUT4 transporters to the PM fractions, accompanied by a decrease in the intracellular membrane fractions. Interestingly, exercise similarly increased GLUT4 content in the PM fractions, however failed to decrease GLUT4 levels in the intracellular fractions. Moreover, both insulin and exercise utilize different signaling pathways, suggesting that two distinct pools of GLUT4 containing vesicles are transported from perinuclear regions to the sarcolemma and T-tubules [92] in response to insulin and contraction. The additive effects of exercise and insulin treatment on glucose uptake by rat skeletal muscle support this two-pool concept [93, 94]. The initiation of insulin- and contraction-stimulated GLUT4 vesicle mobilization involves two distinct molecular signaling pathways that partially converge in their distal aspects, e.g. TBC1D1, TBC1D4 [95-101] and Rac1 [102].

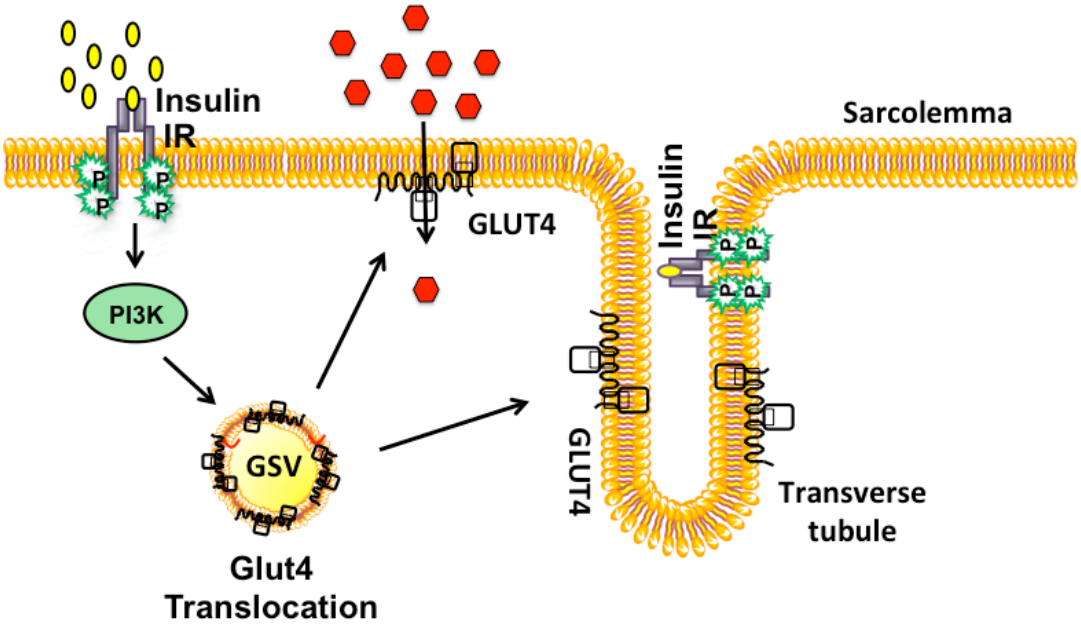


Figure 1. 1. Translocation of GLUT4 to sarcolemma and transverse tubules of skeletal muscle. Exercise induces a surge in intracellular calcium concentration and AMP/ATP ratio, which leads to the activation of AMPK. Activation of AMPK results in the activation of at least two pathways that lead to GLUT4 translocation: 1) activation of the small family Rho GTPase, Rac1, which facilitates actin remodeling-induced GLUT4 vesicle mobilization; 2) the phosphorylation and inactivation of TBCID1, and subsequent activation (via GTP loading) of Rab proteins. Possibly linked wth the Rac1 pathway, Myo1c, an actin-based motor protein, has also been shown to be a novel mediator of both insulin as well as contraction-induced glucose uptake in skeletal muscle [103].

1.4.1.3. Glucose transport/ uptake into skeletal muscle:

Three processes that dictate contraction-induced glucose uptake/ transport into skeletal muscle include glucose delivery [104-106], glucose transport and the metabolism of glucose after its entry into the cell. Exercise-induced increase in blood flow to the skeletal muscle results in net increase in glucose uptake, due to the increased glucose and insulin delivery to the skeletal muscle, along with recruitment of capillaries which increases the surface area for glucose delivery. Moreover, glucose transport/ uptake into the cell depends on the cell surface GLUT4 levels, which requires the translocation and fusion of GLUT4 from intracellular sites to the sarcolemma and T-tubules (detailed in section 1.4.1.2). Once glucose enters into the cell it undergoes immediate phosphorylation to glucose-6-phosphate (G-6-P) due to the action of Hexokinase II (HKII).

1.4.2. Insulin-dependent glucose transport and Glucose transporter translocation in skeletal muscle

Insulin mediates both metabolic and mitogenic responses to control diverse processes including cellular growth, differentiation, apoptosis, lipid, protein and glucose synthesis and breakdown. Failure to regulate these processes results in profound metabolic consequences, including insulin resistance and T2D. Insulin signals the decrease in hepatic glucose output into the blood stream, coordinate with signaling the increases in GLUT4-dependent glucose uptake into the peripheral tissues, altogether regulating whole body glucose homeostasis. GLUT4 is the major contributor amongst all 14 of the GLUTs for the insulin-induced glucose disposal by peripheral tissues, and the acute insulin-stimulated

increase in surface GLUT4 is the major phenomenon required to accomplish this job. The insulin-induced surge in surface GLUT4 into adipocytes is achieved by decreasing the endocytosis/ internalization of GLUT4 and increasing the exocytosis/ externalization of GLUT4. However, in skeletal muscle, insulin does not affect the rate of GLUT4 endocytosis. This implicates an elevated rate in GLUT4 exocytosis/ externalization as the primary mediator of the gain in insulin-stimulated surface GLUT4 levels.

1.4.2.1. Journey of GLUT4: from endocytosis to exocytosis:

The increase in cell surface GLUT4 in response to insulin is predominantly due to the increased exocytosis of GLUT4 in skeletal muscle; in adipocytes, this is also impacted, albeit to a lesser extent, by decreased endocytosis into sorting endosomes.

a) Endocytosis – Clathrin-mediated endocytosis and cholesterol-dependent endocytosis are the two major endocytosis pathways of GLUT4 [107]. Although historically it was assumed that clathrin was the major operating pathway in skeletal muscle cells, a study in L6 myoblasts demonstrated that depletion of cholesterol could exert a notable inhibition of GLUT4 internalization and endocytosis [107], lending support for cholesterol-dependent endocytosis as a required process in skeletal muscle cells. Regarding the mechanisms involved in clathrin-mediated endocytosis [108], Adaptor Protein 2 (AP2) binds to GLUT4 and recruits clathrin to the PM, where it packages GLUT4 into clathrin-coated vesicles, and these are pinched off the membrane with the help of Dynamin. Newly

formed vesicles/ early endosomes at the PM that carry GLUT4 are transported to the cell interior by microtubule-based motor protein, Dynein. Dyein is attached to the GLUT4-containing vesicles via Rab5 (Fig. 1-1). Endocytosed vesicles fuse with the sorting endosomes that are either subjected to degradation or recycling. The GLUT4 transporter can be recycled back to the plasma membrane by budding off into GLUT4 storage vesicles (GSV) from recycling sorting endosomes with the help of small Rho family GTPases specifically Rab 4, Rab 5, Rab 11 and RalA. Insulin causes a 10-40 fold increase in surface GLUT4 by facilitating GLUT4 exocytosis from GSVs and sorting endosomes. In contrast, GSVs can be directed to alternate intracellular regions, where they undergo futile cycles between GSV and the trans-Golgi network (TGN); this aspect is activated by Rab31 and occurs in the absence of insulin. GLUT4, along with insulin-regulated amino peptidase (IRAP), sortilin and low-density lipoprotein receptor-related protein-1 (LRP1), forms a multimeric protein complex that is packaged into a releasable GSVs. Insulin stimulation triggers the inactivation of Rab31 coordinate with the activation of the small GTPase, ARF6 to increase GSV release from the endosomal recycling compartment.

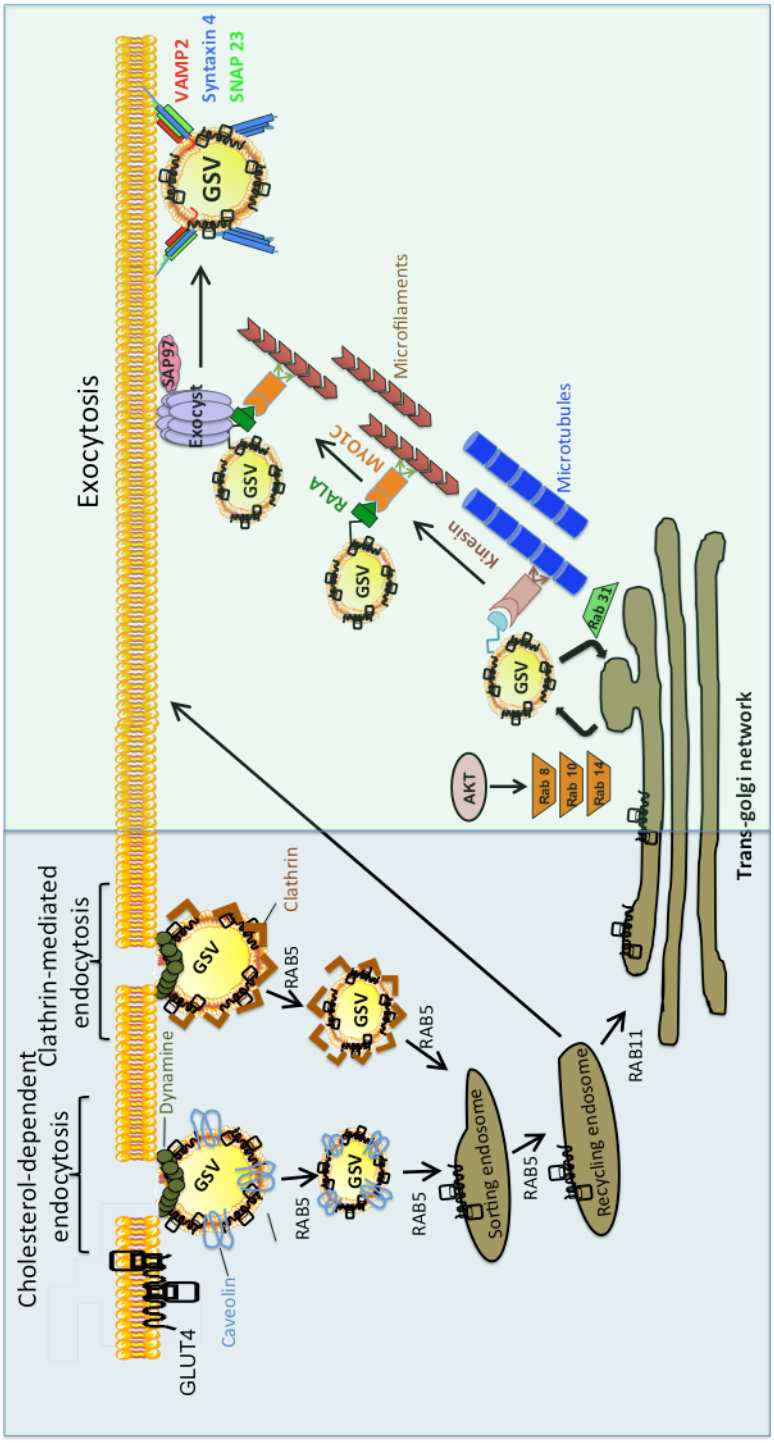


Figure 1.2. Endocytosis and Exocytosis of GLUT4. Schematic model showing clathrin and cholesterol-mediated endocytosis as well as microfilament and microtubule-dependent exocytosis of GLUT4 in Insulin-responsive tissues.

b) Exocytosis – The exocytosis of GSVs requires their translocation to the PM, docking at PM sites, and fusion with the PM. Translocation of GSVs is proposed to initiate in response to the insulin signal triggering release of GSVs from TUG (Tether containing a ubiquitin regulatory X (UBX) domain of GLUT4). Inducing endoproteolytic cleavage of TUG, liberates intracellularly-sequestered GSVs from a retention pool, permitting their translocation to the PM [109, 110]. In skeletal muscle, this involves the activations of small GTPases Rab8 and Rab14. GSVs, like most other secretory vesicles, utilize the actin-based myosin for translocation across short distances (i.e. transport or GSVs less than 1 μm to the PM [110].. GSVs use microtubule-based kinesins for longer distances from perinuclear regions to the PM. Myosin and kinesin motor proteins implicated in this type of process include MYO5A and MYO1C, and KIF3 and KIF5A, respectively. While adipocytes have been shown to use both kinesin- and myosin-based motor proteins to transport GSVs, skeletal muscle cells predominantly use actin-based myosin motors (detailed in section 1.5). In skeletal muscle, the myosin-motor protein MYO1C recognizes the small GTPase RalA present on the GSV membrane, and transports the GSV on the actin cytoskeletal tracks to the docking/targeting site on the PM. In adipocytes it has been further elucidated that GTP-loaded RalA transports GSV to the targeting site via its interaction with exocyst subunits, SEC5 and EXO84. The exocyst subunits are part of a larger exocyst complex, composed of eight distinct

proteins including Sec3, Sec5, Sec6, Sec8, Sec10, Sec15, Sec4 and Exo70. This exocyst complex is stabilized at the PM by the insulin-induced activations of AKT and the small GTPase TC10, along with the action of the lipid raft-localized synapse-associated protein 97 (SAP97). Subsequent to this docking step, the protein kinase C (PKC)-dependent phosphorylation of the protein SEC5 inhibits the interaction of RalA and the exocyst complex, disengaging the GSV from the targeting/docking machinery to prepare the GSV for fusion with the PM. GSV fusion requires SNARE complexes composed of the GSV-based vesicle-SNARE vesicle associated membrane protein-2 (VAMP2), and two PM-localized target-SNARE proteins, Syntaxin 4 and synaptosomal-associated protein 23 (SNAP-23). Docking and fusion of GSVs also requires the recruitment of double C2-like domain containing protein β (Doc2β) to the PM. Doc2β sequesters the regulatory protein Munc18c and facilitates activation/ opening of Syntaxin 4, which once opened, participates in the SNARE complex formation leading to GSV exocytosis.

1.5. THE ROLE OF THE ACTIN CYTOSKELETON IN GLUT4 EXOCYTOSIS

Signaling to and consequent sorting of GSVs from the intracellular regions of the muscle cell to the docking/fusion sites at the PM requires spatiotemporal regulation of components of the dynamic cytoskeleton. In adipocytes, multiple studies using disrupting agents of either KIF3 or KIF5b have suggested both actin- and microtubule-based cytoskeletal regulations are required for the insulin-induced mobilization of GSVs to the PM [111]. Contrary to this concept,

disruption of microtubule-based motors using the pharmacological agent colchicine in skeletal muscle cells (L6 muscle cells) failed to impair the insulin-induced GSV translocation to the PM[112]. Supporting the actin-based mobilization concept, treatment of L6 cells with the inhibitor of actin branching, Swinholide A, blunts GLUT4 translocation and membrane ruffling, [112, 113]. Moreover, depolymerization of filamentous actin (F-actin) by the actin monomer sequestering agent, Latrunculin B, exhibits a time- and concentration-dependent decline in glucose transport into muscle cells, implicating the requirement of an intact F-actin network for insulin-induced GLUT4 translocation and glucose uptake [114]. Interestingly, knockdown of Myo1c in L6 muscle cells disrupted actin filaments, while Myo1c overexpression immobilized those GSVs close to the PM (as detected by total internal reflection fluorescence, TIRFM). As such, a prevailing concept is that the interaction of vesicular Myo1c with F-actin regulates GLUT4 vesicle tethering to the actin cytoskeleton and subsequent insulin-induced GLUT4 vesicle fusion to the PM [115].

1.5.1. Insulin-induced F-actin remodeling and GLUT4 vesicle translocation

Cytoplasmic actin exists in 2 forms: a) monomeric globular actin (G-actin) b) filamentous actin (F-actin). F-actin remodeling encompasses the process of continuous cycles of polymerization of actin at the barbed end of the existing filament, and depolymerization of F-actin to G-actin at the pointed end. Both F-actin nucleating proteins, such as those of the Arp2/3 complex, with the opposing actions of actin severing proteins such as cofilin and gelsolin, tightly regulate this

F-actin remodeling process [116-118]. Importantly, multiple studies have shown that the insulin-stimulated remodeling of F-actin, as opposed to simply its polymerization, is essential for the translocation of GSVs to the PM of skeletal muscle cells [119, 120].

1.5.1.1. Actin polymerizing proteins:

Several actin nucleating/ branching proteins function together to regulate the intricate process of actin polymerization, of which the Arp2/3 complex, a branched actin filament nucleator, is implicated specifically in GLUT4 vesicle translocation in skeletal muscle cells. Arp2/3 is a 220 kDa complex consisting of seven subunits (ARP2, ARP3, ARPC1-5). From a structural perspective, the actin-related proteins (ARP2 and ARP3) resemble monomeric actin because together they seem to mimic an actin dimer, initiating the formation of new actin branches on existing F-actin filaments. Knockdown of ARP3 or ARPC2 subunit of ARP2/3 complex in skeletal muscle cells abrogated insulin-induced F-actin remodeling and GLUT4 translocation to the PM [121]. Intriguingly, PAK1, a well studied downstream effector of Rac1 that is involved in insulin-induced cortical actin remodeling, phosphorylates ARPC1, a regulatory subunit of the ARP2/3 complex, to initiate actin remodeling in a mammalian breast cancer cell line (MCF-7) [122]. This suggests that PAK1 may act via the regulation of ARP2/3 complex in skeletal muscle cells. Although the ARP2/3 complex can initiate branched actin filament nucleation, it requires the actions of nucleation promoting factors, such as N-WASP, WAVE and Cortactin, to stimulate its activity and carry out actin polymerization.

a) Nucleation promoting factors (NPF) – NPFs are classified into two classes: Type I NPFs interact with monomeric actin molecules, whereas Type II NPFs bind to the actin filaments. Recent findings by Nakaya et al [123] show that overexpressing Cortactin, a type II NPF, in L6-GLUT4myc cells increased insulin-induced GLUT4 translocation. Furthermore, knockdown of Cortactin completely blunted insulin-dependent glucose uptake into L6-GLUT4-myc myotubes. While these data clearly show a requirement for Cortactin in GLUT4 vesicle mobilization, Cortactin is considered to be a relatively weak activator of the Arp2/3 complex, and requires strong NPFs such as neural Wiskott Aldrich Syndrome Protein (N-WASP, also a type II NPF) to accomplish Arp2/3 mediated actin polymerization. Supportive of a role for N-WASP in muscle cells, Brozinick *et al* showed that N-WASP localized to F-actin in an insulin-dependent manner, and that this was abrogated by disruption of F-actin by Latrunculin B [114] in mature skeletal muscle. In non-muscle cells, PAK1 has been shown to use Cortactin as a substrate, phosphorylating serines 405 and 418, which increases its association with N-WASP and facilitates the polymerization of actin during clathrin-independent endocytosis [124]. While this would serve as a means to connect GSV mobilization via ARP2/3 and N-WASP with Cortactin, this has yet to be tested in skeletal muscle cells. To date, there are no reports implicating the role of any known type 1 NPFs in the mobilization of GSV to the skeletal muscle cell surface.

b) Actin stabilizers – Polymerizing actin is also stabilized by proteins such as Fodrin, also known as nonerythroid spectrin. Interestingly, Fodrin has been shown to interact with the t-SNARE syntaxin 4 in response to insulin, and hence is implicated in the fusion of GSVs with the PM in rat adipocytes [125]. Moreover, the Calpain-induced cleavage of Fodrin at the sarcolemma in muscle is associated with Duchenne Muscular Dystrophy (DMD) [126], a disease wherein insulin resistance is an early manifestation [127]. Increased cleaved fodrin is also found in pancreatic β cells of T2D subjects, further linking fodrin to metabolic control mechanisms in glucose homeostatic tissues/cells.

c) Actin crosslinking proteins – Filamin A is an actin crosslinking protein that can be phosphorylated by PAK1 at Ser2152 and was shown to be involved in PAK1-dependent membrane ruffling in mammalian breast cancer cells [128]. Once phosphorylated, Filamin A interacts with the GTPase binding domain of PAK1, inducing its further activation and creates a feed-forward loop of activation of PAK1. In skeletal muscle, cross-sectional analyses show the colocalization of Filamin A with phosphoinositidylinositol 3,4,5 trisphosphate (PtdIns[3,4,5]P_3) 5-phosphatase (SHIP-2) in the membrane ruffles, and these are considered to be the sites of insulin-induced GLUT4 translocation. Taken together, these findings suggest a possible mechanism involving PAK1 with Filamin A and SHIP-2 in GSV mobilization in skeletal muscle [129].

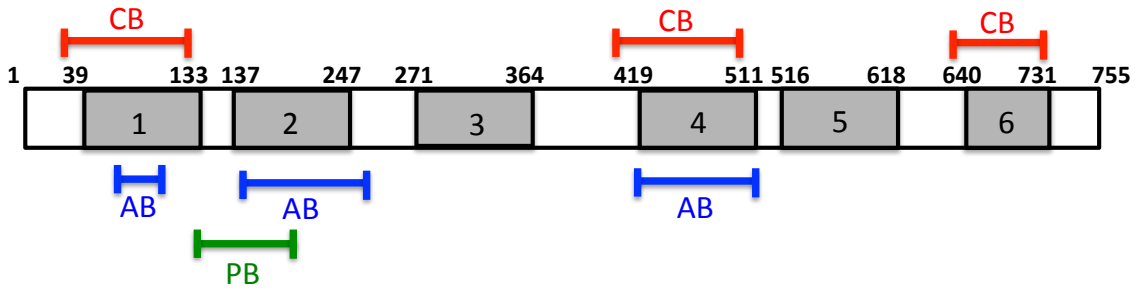
1.5.1.2. F-actin severing/ depolymerizing and capping proteins:

Actin depolymerizing factor (ADF)/ Cofilin and Gelsolin are the two major actin-severing proteins implicated in the regulation of the skeletal muscle actin filament network [121]. In non-muscle cells, Cofilin phosphorylation at Ser3 by LIM-kinase (LIMK) inhibits Cofilin's actin severing ability [130]. In contrast, Cofilin's dephosphorylation at Ser3 by the phosphatase Slingshot (SSH) will activate Cofilin [131]. Recent findings by Wang *et al* [132]reveal the requirement of PAK1 for the insulin-dependent dephosphorylation of phospho-Cofilin in skeletal muscle. Interestingly, PAK4 a Group II PAK member, has been shown to activate LIMK and inactivate SSH via phosphorylation *in vitro*, suggesting a plausible role in the inactivation of Cofilin [133]. However, the involvement of PAK4 as a potential regulator of Cofilin activity in skeletal muscle, rather *in vivo* is yet to be explored.

Gelsolin, is a member of the class of actin severing proteins that mediate calcium-dependent severing and capping of actin filaments at their barbed ends. Gelsolin consists of six structurally similar domains (G1-G6) [134] and assumes an autoinhibitory form in the absence of activation, due to the intramolecular interaction of its C-terminal tail with the G2 domain (Fig. 1-2). Mechanistically, calcium binding to Gelsolin relieves it from the autoinhibitory state and increases its affinity for the ADP-associated actin filament to initiate its severing function [135]. Gelsolin is expressed ubiquitously [136], and has been implicated as a regulator of unsoliticed insulin secretion from pancreatic β cells. This concept is supported by a study showing Gelsolin bound to the t-SNARE protein Syntaxin 4 and blocked Syntaxin 4 engagement in active vesicle fusion [137]. Given a

number of similarities in the SNARE mediated exocytosis machinery in the insulin granule exocytosis in pancreatic β cells and GLUT4 exocytosis in skeletal muscle, this carries the potential to be a shared mechanism in skeletal muscle GLUT4 exocytosis.

A second actin capping protein, Tropomodulin3, has been identified as a novel AKT2 interacting partner that is regulated by phosphorylation at Ser71 by AKT2 in 3T3-L1 adipocytes. Knockdown of (Tmod3) abrogated insulin-induced GLUT4 vesicle fusion and subsequent glucose uptake [138]. Moreover, TIRFM analysis of adipocytes showed a requirement for Tmod3 and its phosphorylation in insulin-initiated actin remodeling. All together, the studies of these actin modifying factors link their actions to F-actin cytoskeletal reorganization and GLUT4 vesicle translocation/ exocytosis to the cell surface of insulin-sensitive tissues.



CB: Ca^{2+} binding domain
AB: Actin binding domain
PB: Phosphatidylinositol (3,4,5) triphosphate (PIP3) binding domain

Figure1.3. Schematic representation of Gelsolin protein (1-755 amino acids)

1.6. THE CANONICAL INSULIN SIGNALING PATHWAY IN SKELETAL MUSCLE

The classic insulin signaling pathway in skeletal muscle cells initiates with insulin binding to the insulin receptor (IR) present on the extracellular surface of the skeletal muscle cell. Insulin binds to the extracellular α subunit of the tetrameric insulin receptor, which elicits a conformational change in the α subunit and receptor autophosphorylation at several tyrosine residues in the intracellular β subunit [139]. These phosphorylation events induce conformational changes in the intracellular IR subunits to further increase the catalytic kinase activity of the IR, resulting in the recruitment of its receptor substrates. Recruited IR substrates in this pathway include insulin receptor substrate (IRS) via pleckstrin homology (PH) domains and phosphotyrosine binding domains (PTB) in the IRS amino termini. In skeletal muscle, IRS-1, but not IRS-2 plays a critical role in myoblast differentiation and glucose metabolism[140, 141]. Downstream of IRS-1, the pathway branches into at least two major transduction routes, one in which upon tyrosine phosphorylation IRS-1 protein binds Src-homology-2 (SH2) domain containing proteins including p85, the regulatory subunit of class I Phosphatidylinositol-3 kinase (PI3K) and targets the enzyme to the membrane. At the membrane, PI3K phosphorylates phosphatidylinositol 4,5-biphosphate (PIP2) to produce the lipid second messenger phosphatidyl 3,4,5-triphosphate (PIP3) that recruits several PIP3 binding proteins to the membrane. In the other pathway, phosphorylated IRS-1 interacts with adaptor molecule, growth factor receptor bound protein 2 (GrB2) and son-of-sevenless (SOS) complex activating

Ras-MAPK cascade and mitogenesis. The IRS-1-PI3K pathway, which mediates insulin's action on glucose metabolism, further bifurcates into at least two parallel signaling pathways downstream of PI3K (Fig. 1-3).

1.6.1. The PI3K-AKT-AS160 signaling arm

The insulin-stimulated recruitment of p85 by IRS1 brings p110, the catalytic subunit of PI3K to the PM, where it catalyzes the phosphorylation of the 3' position on the inositol ring of the phosphoinositide (PI) lipids[142].. PI3K specifically catalyzes formation of PIP2 from PI(4)-phosphate; the PIP3 generated from PIP2 then aides in the recruitment and activation of PIP3-binding proteins that contain pleckstrin homology (PH) domains, namely phosphoinositide-dependent-kinase 1 (PDK1) and AKT (protein kinase-B). Of the three AKT/PKB isoforms (AKT1, 2 and 3), AKT2/PKBβ has been shown to be essential for glucose transport into skeletal muscle, using whole body KO rodent models [143] as well as functional studies in skeletal muscle with *in vivo* electrotransfer of constitutively active-AKT2 (ca-AKT2) [144]. PIP3-guided PM recruitment of PDK1 and AKT2 aide in the phosphorylation and activation of $AKT2^{T308}$ and of PDK1 and PDK2 (also known as mammalian target of Rapamycin complex 2[mTORC2]) at S473 [145, 146]. Fully activated AKT2 phosphorylates one of its substrates, the Rab guanine nucleotide activating protein (GAP) called $AS160^{T642}$ (also known as TBC1D4).

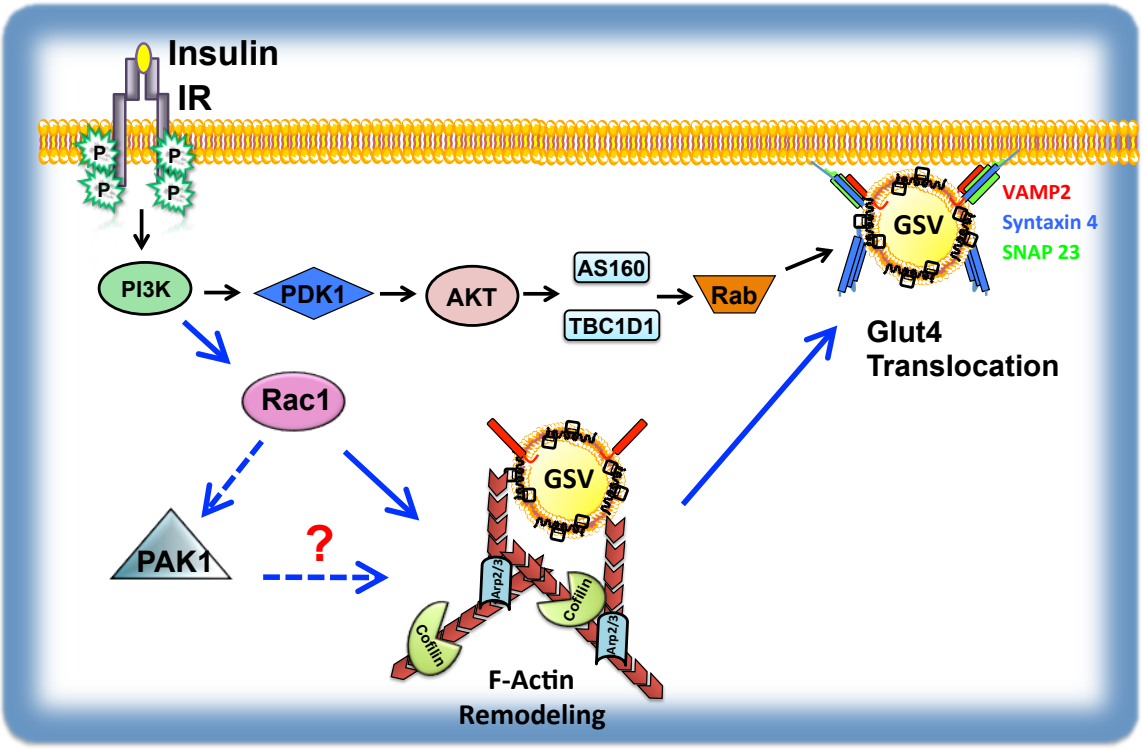


Figure 1.4. Insulin signaling pathway. Insulin binding to the insulin receptor (IR) and results in the activation of PI3K, downstream of which the pathway bifurcates into at least two signaling pathways: 1) Activation of Phosphoinositide dependent kinase-1 (PDK1) allows PDK1 to access and phosphorylate AKT. Activated AKT in turn phosphorylates ad inactivates Rab GTPase activating proteins AS160 and TBCID1, thus facilitating Rab.GTP mediated GLUT4 storage vesicle (GSV) translocation; 2) Activated Rac1 downstream of PI3K recruits its downstream effector PAK1 and initiates cortical F-actin remodeling which is regulated by actin binding proteins Cofilin and Arp2/3 complex. Cortical actin remodeling facilitates GSV translocation and fusion to the cell surface with the help of SNARE complex proteins, VAMP2, Syntaxin 4 and SNAP-23, and results in glucose uptake.

AS160 targets Rab8A and Rab14 in skeletal muscle [147] and inactivates each Rab. Through its GTPase activating domain, AS160 maintains Rab proteins (small G proteins involved in membrane trafficking) in an inactive GDP-bound state under basal/unstimulated conditions [148]. Upon dissociation from AS160, mediated by their phosphorylation and binding by the 14-3-3 protein, Rab proteins are switched to their active GTP-bound state to facilitate GSV trafficking to the PM. Interestingly, AS160 is also regulated by the action of AMPK, which is a key regulator of the contraction-induced glucose uptake pathway [99], placing AS160 at a point of convergence linking insulin-and contraction–mediated signaling (detailed in section 1.3.1.) to facilitate glucose entry into skeletal muscle.

1.6.2. Rac1-PAK1-actin remodeling pathway

In the last decade much emphasis has been placed on delineating the alternative pathway involving small Rho family GTPases, a pathway found to proceed in parallel to that of the AKT-AS160 pathway, downstream of PI3K. Rac1 (Ras-related C3 botulinum toxin substrate 1) is a small Rho family GTPase that acts as a molecular switch to execute diverse cellular processes including cell cycle, cell-cell adhesion and actin cytoskeleton regulated motility, by changing between active GTP-bound form and inactive GDP-bound form. Recently, Rac1 has been shown to function downstream of PIP3, in the regulation of insulin-dependent GLUT4 vesicle translocation and glucose uptake into skeletal muscle. In skeletal muscle cells, the activation of Rac1 transmits signals to various downstream effectors involved in cytoskeletal remodeling. Although both AKT and Rac1 act downstream of PI3K [149], signaling pathways followed by each protein were

suggested to take parallel and independent routes to facilitate insulin-dependent glucose uptake in skeletal muscle. This was supported by multiple studies, using dominant negative mutants and pharmacological/chemical inhibitors of AKT [150], none of which showed any effect upon insulin-induced actin cytoskeletal remodeling. Consistently, neither Rac1 knockdown nor expression of the constitutively active Rac1 mutant altered insulin-stimulated AKT phosphorylation [139]. However, more recent studies by Takenaka *et al* using skeletal muscle specific Rac1 KO mice demonstrate significant reduction in AKT2 or PI3K dependent GLUT4 translocation in skeletal muscle, contradicting the above findings [151]. Despite this discrepancy, studies using both cell culture models as well as whole body KO mouse models have implicated Rac1 and its downstream signaling to p21-activated kinase 1 (PAK1) to be crucial regulators of both insulin- and contraction-induced cytoskeletal remodeling as a key element in regulated GLUT4 vesicle trafficking.

1.6.2.1. Structure, activation and function of PAKs:

PAK proteins are serine/threonine kinases that consists of 6 family members divided into two groups; Group I PAKs include PAK1, 2 and 3, Group II PAKs consist of PAK4, 5 and 6. The basic structure of Group I PAKs: each consists of an N-terminal regulatory domain, which includes a GTPase binding domain (GBD) that is also known as a Cdc42-Rac Interactive Binding (CRIB) domain, overlapping with an auto-inhibitory domain (AID), and a C-terminal kinase domain. Group I PAKs exist in an inactive homodimer conformation (unlike Group II PAKs, which exist as monomers), where the AID domain of one

monomer is bound to the kinase domain of the other. Activation of group I PAKs occurs upon binding of small GTPases Cdc42 or Rac to the GBD, relieving the inhibitory conformation, and triggering the autophosphorylation at the activation loop for subsequent activation of PAK's kinase activity (Fig. 1-4). Group II PAK activation differs from that of Group I PAKs, since the AID of Group II PAKs is thought to allosterically modify the constitutively phosphorylated kinase so that it becomes active [152]. Both group I and II PAKs are important regulators of various biological processes including cell motility, survival, proliferation, cytoskeletal organization as well as transcription and translation. Owing to their involvement in the above physiological processes, deregulation of functional PAKs are implicated in multiple disorders such as cancer, mental retardation, allergy, inflammatory and cardiovascular diseases [153].

1.6.2.2. PAK1 structure and domains:

a) Proline-rich regions – Interestingly, PAK1 also interacts with multiple substrates depending on unique cellular conditions through its five proline-rich regions, which contain PXXP (where P and X represent Proline and any amino acid, respectively) sites that resemble Src Homology 3 (SH3) domain binding motifs. Nck, an adaptor protein that contains one SH2 domain and three SH3 domains, interacts with the first proline-rich SH3 domain binding motif (aa 12-18) of PAK1 through its second SH3 domain, linking PAK1 and receptor tyrosine kinases (such as IR) in cell proliferation and growth [154].

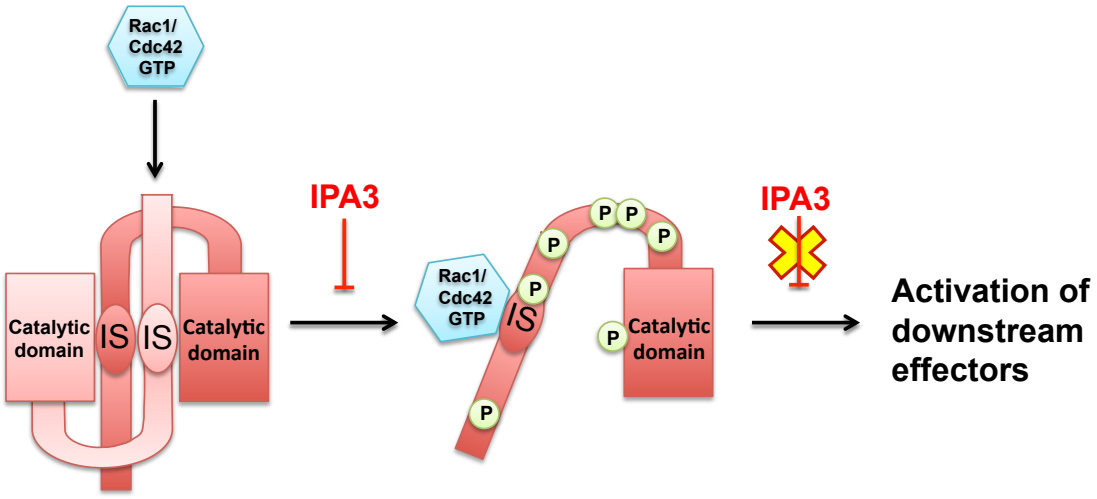


Figure 1.5. PAK structure and activation by small Rho GTPases. Under basal conditions PAK exists as a homo-dimer in an auto-inhibitory conformation which upon stimulation by growth factors opens up into an active monomeric conformation due to binding of activated small Rho GTPases such as Rac1/ Cdc42-GTP and auto-phosphorylation of PAK on several serine and threonine residues.

Similarly, PAK1-Grb2 (Growth factor receptor bound protein 2) adaptor protein associate via the second SH3 binding motif of PAK1, mediating the coupling of activated Epidermal Growth Factor Receptor (EGFR) and PAK1 upon Epidermal Growth Factor (EGF) stimulation in HaCaT cells (an immortalized human keratinocyte cell line). In this manner, the upstream receptor kinases and downstream PAK1 signaling are linked [155]. Moreover, PAK1 binds to the guanine nucleotide exchange factor (GEF) for Cdc42, PAK-interactive exchange factor/cloned out of library (PIX/COOL) protein through a non-canonical proline-rich region (aa 182-203).

b) Autoregulatory fragment – The N-terminal region of PAK1 from amino acids 70-149 is termed as the autoregulatory fragment. This region consists of three main segments. The first, a p21-binding domain (PBD; aa 70-113), includes the minimum sequence for Cdc42/Rac interactive binding (CRIB; aa 75-90), essential for the binding of small GTPases. Studies using site-directed mutants and truncated fragments revealed the sequences required for the high-affinity binding of PAK1 to Cdc42 and Rac1 [156-158]. The second region, an autoinhibitory domain (AID; aa 83-149), acts as an inhibitory switch (IS) and is essential for the *trans*-autoinhibition of group I PAKs and the mechanism of inhibition is elaborated in the sections 1.6.2.1. [159]. The C-terminal extension of the IS region sequesters the critical element, named the kinase inhibitor (KI; aa 136-149) segment, which passes through the cleft of the kinase

domain, and is required for the “autoinhibited state of PAK”. A segment of PAK1 containing the deletion of amino acids 81-87 in the N-terminal region of the IS motif, termed as Dimerizatin (Di; aa 81-87) segment, exhibits a decreased dimerization [160], implicating an importance of trans-dimerization for the inhibition of PAK1 under basal conditions. Binding of activated Cdc42 or Rac to the CRIB/PBD region relieves the autoinhibited state of the PAK1 dimer by disrupting the intermolecular interactions between the IS domain of one monomer and the KD of the other (details of the activation process are described in the next section).

c) Kinase Domain – The kinase domain (aa 249-545) is the catalytically active site of PAKs that has the standard, two-lobe structure, and is characteristic of all the protein kinases [161]. Interestingly, PAK1 contains an intrinsically active kinase domain that catalyzes the serine/threonine phosphorylation of its substrates, even in the absence of activators. This unique feature advocates the requirement of tight regulation of the active site to avoid unwarranted activation of kinase function. Notably, a stretch of 30 residues in the large lobe of the kinase domain, designated as the “activation loop” (A-loop), contains the critical phosphorylation site Threonine 423 (T423), required for the complete activation of PAK1 [162]. Moreover, evidence for the existence of the kinase domain in an essentially active conformation, even in the absence of T423 phosphorylation, stems from the studies using kinase-dead mutants (K229R) and phosphomimetic (T423E) mutants of PAK1 [163]. At the end

of the C-terminus of the Kinase domain, PAK1 has a binding site for Gβγ subunit of heterotrimeric G-proteins, which are implicated in the inhibition of PAK1 kinase function [164, 165].

1.6.2.3. Multistage activation of *trans*-autoinhibited PAK1:

PAK1, also referred as PAKα, was first discovered in 1994 in rat brain as a protein kinase that binds to the active GTP-bound form of small GTPases Cdc42 and Rac1 [166, 167]. Originally named PAK65, PAK1 is the most extensively studied protein among the PAKs, and is highly homologous to the yeast protein Ste20p. The activation of PAK1 is a multistage process that starts with the binding of GTP-bound form of Cdc42 or Rac to PAK1's GBD at the N-terminal region, which triggers a series of conformational changes involving disruption of the PAK1 dimer and rearrangement of kinase domain into a catalytically competent state. Binding of activated Cdc42 or Rac induces conformational changes in the GBD and unfolds the IS domain, which results in the dissociation of the KI fragment (within the AID) from the catalytically active kinase domain. However, PAK1 assumes its fully activated state only after phosphorylation of T423 in the activation loop and subsequent phosphorylation of serine residues at 21, 57, 144, 149, 198 and threonine residue at 212 [160], within the KI region and the IS domain, preventing its redimerization into an inactive state [159] The significance of the T423 site in the activation of PAK1 comes from the studies showing reduced kinase activity of PAK1 carrying the T423A mutation [168]. However, signaling downstream of PAK1 that leads to GLUT4 translocation in skeletal muscle is still unexplored.

1.6.2.4. PAK1 as a signaling kinase:

Since its discovery in 1994, several cytoskeletal proteins, adaptor proteins, Guanine exchange factors (GEF), integrins, kinases as well as phosphatases have been identified as PAK1 substrates. PAK1 notably signals downstream to pathways involved in cytoskeletal remodeling. For example, the ARP2/3 complex, Filamin A, Cortactin, Cofilin and LIMK are some of the major regulators of the actin cytoskeletal network that can be regulated by phosphorylation by PAK1 at unique serine/threonine residues. These are discussed in detail in Section 1.4. In addition to modulating proteins involved in the actin cytoskeletal network, PAK1 also regulates microtubule dynamics by phosphorylating Tubulin cofactor B (TCoB), a cofactor in the assembly of α/β-tubulin heterodimers at Serine residues 65 and 128. Although, studies using agents that inhibit microtubule polymerization such as colchicine suggested against a requirement for microtubule-mediated regulation of insulin- or contraction-stimulated glucose transport in skeletal muscle [112], more recent findings (Schisler (2016 FASEB) using mice deficient in Carboxyl terminus of HSC70-Interacting Protein (CHIP) revealed the significance of microtubule dynamics for insulin-dependent GLUT4 translocation in skeletal muscle. Decreased GLUT4 translocation was accompanied by reduced microtubule polymerization and phosphorylation of Stathmin (at Ser16), a microtubule-regulating protein in the CHIP-depleted skeletal muscle cells. Intriguingly, PAK1 phosphorylates stathmin at Ser16 and regulates its activity in Hep2 cells [169]. Altogether, the above findings point towards a potential role of PAK1 in the regulation of microtubule-dependent

GLUT4 trafficking in skeletal muscle. Whether decreased phospho-Stathmin (S16) levels associate with decreased PAK1 activity or phospho-PAK1 levels in the CHIP-depleted skeletal muscle cells remains an open question.

1.6.2.5. PAK1 as a scaffolding protein:

Although a vast number of phospho-proteins have been identified as PAK1 substrates, increasing evidences using kinase-dead mutants of PAK1 indicate a kinase- independent scaffolding function for PAK1 in the regulation of cytoskeletal reorganization and cell motility [155, 162]. As a scaffold, PAK1 signals to downstream effectors not by mere transfer of a phosphate group, but by creating a platform to allow the tethering of multiple members of a signaling pathway into complexes. Towards this, the PAK1-PDK1-AKT signaling pathway has been very well studied. Briefly, PAK1 acts a scaffold to facilitate the complex formation between AKT and PDK1, which assists in the phosphorylation of AKT at T308 by PDK1 [170]. Interestingly, it has been shown that PAK1 is phosphorylated and activated by AKT and PDK1, suggesting a positive-feedback loop between PAK1 and PDK1/AKT to regulate downstream signaling pathway.

PP2A is a serine protein phosphatase that is involved in glucose transport and glycogen synthesis [171, 172]. PP2A has been shown to associate with and subsequently activate the actin-severing protein Cofilin, by dephosphorylating Cofilin at Ser3 [173]. Cofilin dephosphorylation has been shown to be a critical step involved in the insulin-stimulated Rac1-PAK1 pathway that leads to GLUT4 translocation in skeletal muscle. PAK1, through its scaffolding function, induces PP2A dephosphorylation at Y307 to thereby increase its phosphatase activity

[174]. Intriguingly, a study using whole body PAK1 KO mice implicated a requirement for PAK1 in the activation of Cofilin in skeletal muscle [132]. Hence, one possible explanation for the mechanism of cofilin activation by PAK1 would be that PAK1 scaffolds and activates PP2A, leading to the dephosphorylation and subsequent activation of cofilin, which in turn regulates the actin remodeling process and facilitates the insulin-dependent GLUT4 translocation in skeletal muscle. Alternatively, PAK1 might activate cofilin via its signaling function by regulating SSH1/ Chronophin (a second potential Cofilin phosphatase) phosphatase activity [175]. Further investigation is warranted to fully understand this process.

1.6.2.6. Regulation of PAK1 activity:

a) Activators of PAK1 – GTP-loaded Small Rho family GTPases such as Rac, Cdc42 and TC10 activate PAK1 by binding to the GBD/ CRIB domain at the N-terminal region, relieving the autoinhibited homodimer conformation, to activate the monomer form (detailed description in earlier section 1.6.2.2.). While Cdc42/Rac1 and TC10 function in pancreatic β cells and adipocytes, respectively, Rac1 is the only isoform among Rac1, Rac2 and Rac3 that regulates both the insulin- and the contraction-stimulated glucose uptake into skeletal muscle [102, 176]. Besides the small Rho family GTPases, Wnt-1 responsive Cdc42 Homolgue (Wrch-1), an atypical small Rho GTPase regulated by Wnt-1 signaling, also activates PAK1, although the mechanism of activation is not fully understood [177].

Moreover, Phosphatidylinositol 4,5-bisphosphate (PIP2), the most abundant phosphoinositide at the PM, acts together with Cdc42 to synergistically activate PAK1 by binding to the basic region and the GBD in the autoinhibitory domain of PAK1, respectively. This recruits PAK1 to the membrane ruffles at the plasma membrane [178]. PIP3, the second messenger and the phosphorylated form of PIP2, recruits PH domain-containing proteins and regulates the insulin-stimulated GLUT4 trafficking in peripheral tissues. However, the significance of PIP2 in the regulation of insulin-stimulated GLUT4 vesicle exocytosis remains elusive.

The recruitment of PAK1 to membranes also leads to its activation. SH3 domain-containing adaptor proteins such as Nck/Grb2 bind to the proline-rich SH3 binding motif of PAK1, recruiting it to the PM and assisting in the activation of PAK1 by small Rho GTPases [154]. Moreover, Nck has been shown to bind to the activated IRS-1 via its other SH2 binding motif in response to insulin. Together, these data might suggest that Nck, by activating PAK1, might mediate one of the plausible mechanisms to couple the upstream insulin signaling events involving the cell surface receptors and its downstream effectors, required for GLUT4 exocytosis.

b) Negative regulators of PAK1 – Since uncontrolled PAK1 activation might result in deleterious metabolic effects, mechanisms to ensure appropriate PAK1 activation are essential. Recent evidence from Takenawa *et al* revealed that Skeletal muscle and kidney-enriched inositol polyphosphate phosphatase (SKIP), a protein already implicated in insulin

signaling in skeletal muscle, negatively regulates PAK1 by blocking its scaffolding function in regulating PDK1-AKT2 mediated insulin-stimulated glucose uptake.

1.7. ROLE OF PAK1 IN THE MAINTENANCE OF WHOLE BODY GLUCOSE HOMEOSTASIS

Whole body PAK1 KO mice exhibited glucose intolerance with impaired insulin sensitivity, underpinned by defects in insulin-stimulated GLUT4 vesicle translocation and cofilin dephosphorylation in skeletal muscle. As a whole-body KO model, the PAK1 KO mice also exhibited defects in glucose-induced insulin secretion. In an independent study of the whole body PAK1 KO mice, a significance for PAK1 in mediating the cross talk between insulin and Wnt signaling pathways on gut and brain proglucagon expression was raised, a route completely different from pancreas and skeletal muscle. The Insulin Resistance Atherosclerosis Study (IRAS) and Genome-Wide Association Study (GWAS) have localized susceptibility genes, including PAK1, to Chromosome 11, a notable diabetes locus. Substantiating this, PAK1 protein levels were reduced in human T2D islets, implicating the significance of PAK1 in the maintenance of metabolic homeostasis.

Insulin and contraction both stimulate the translocation of perinuclear GSVs and their fusion to the sarcolemma and T-tubules, facilitating the entry of glucose into the skeletal muscle. Interestingly, both insulin and contraction induce the activation of PAK1 in skeletal muscle. Moreover, multiple studies using muscle specific inducible Rac1 KO mice have revealed the requirement of Rac1 in both

insulin and contraction-stimulated glucose uptake into mature skeletal muscle. However, the activation of PAK1, a known downstream target of Rac1 in other cell types, has yet to be placed in the signaling cascade emanating from Rac1 in muscle cells.

1.8. RATIONALE AND CENTRAL HYPOTHESIS

The rationale for studying the role of PAK1 in skeletal muscle insulin sensitivity is that delineating the molecular mechanisms of PAK1 signaling pathway in skeletal muscle glucose uptake will provide us with potential novel therapeutic targets for interventions to treat or even prevent T2D. The central hypothesis of my dissertation is formulated based on published literature including the following evidence: 1) Whole body PAK1 KO mice exhibited glucose intolerance with impaired insulin sensitivity; 2) PAK1 protein levels and function were reduced in metabolically-relevant human T2D tissues, implicating the significance of PAK1 in the maintenance of metabolic homeostasis. Thus my central hypothesis is that, PAK1 exists in an insulin signaling cascade that triggers F-actin remodeling to facilitate GLUT4 vesicle translocation to the cell surface, leading to glucose uptake into the skeletal muscle and clearance of excess circulating blood glucose. To test this hypothesis I pursued two Specific Aims: 1) Determine the role and requirement of PAK1 signaling in insulin-dependent glucose uptake in skeletal muscle cells; and 2) Delineate the PAK1 signaling cascade that evokes F-actin remodeling required for the insulin-stimulated GLUT4 translocation in skeletal muscle cells.

CHAPTER 2

SIGNALING OF THE P21-ACTIVATED KINASE (PAK1) COORDINATES INSULIN-STIMULATED ACTIN REMODELING AND GLUCOSE UPTAKE IN SKELETAL MUSCLE CELLS

2.1. SYNOPSIS

Skeletal muscle accounts for ~80% of postprandial glucose clearance, and skeletal muscle glucose clearance is crucial for maintaining insulin sensitivity and euglycemia. Insulin-stimulated glucose clearance/uptake entails recruitment of glucose transporter 4 (GLUT4) to the plasma membrane (PM) in a process that requires cortical F-actin remodeling; this process is dysregulated in Type 2 Diabetes. Recent studies have implicated PAK1 as a required element in GLUT4 recruitment in mouse skeletal muscle in vivo, although its underlying mechanism of action and requirement in glucose uptake remains undetermined. Toward this, we have employed the PAK1 inhibitor, IPA3, in studies using L6-GLUT4-myc muscle cells. IPA3 fully ablated insulin-stimulated GLUT4 translocation to the PM, corroborating the observation of ablated insulin-stimulated GLUT4 accumulation in the PM of skeletal muscle from PAK1$^{-/-}$ knockout mice. IPA3-treatment also abolished insulin-stimulated glucose uptake into skeletal myotubes. Mechanistically, live-cell imaging of myoblasts expressing the F-actin biosensor LifeAct-GFP treated with IPA3 showed blunting of the normal insulin-induced cortical actin remodeling. This blunting was underpinned by a loss of normal insulin-stimulated cofilin dephosphorylation in IPA3-treated myoblasts. These findings expand upon the existing model of actin remodeling in glucose uptake, by placing insulin-stimulated PAK1 signaling as a required upstream step to facilitate actin remodeling and subsequent cofilin dephosphorylation. Active, dephosphorylated cofilin then provides the G-actin substrate for continued F-

actin remodeling to facilitate GLUT4 vesicle translocation for glucose uptake into the skeletal muscle cell.

2.2. INTRODUCTION

Insulin maintains the glucose homeostasis of the body by mobilizing insulin-responsive glucose transporter 4 (GLUT4)-containing vesicles from intracellular compartments to the plasma membrane (PM) of muscle and adipose cells, facilitating glucose uptake into these tissues [179-181]. Defects and/or deficiencies in insulin-stimulated glucose uptake by these peripheral tissues promote the development of peripheral insulin resistance and pre-diabetes [182]. Skeletal muscle glucose uptake accounts for ~80% of all insulin-stimulated uptake [183]. Under normal insulin-sensitive conditions, circulating insulin binds to the insulin receptor present on the extracellular surface of the skeletal muscle cell to initiate an intracellular signaling cascade which bifurcates downstream of phosphatidylinositol 3-kinase (PI3K) into at least two parallel pathways that lead to GLUT4 vesicle translocation, facilitating glucose uptake into the muscle cell [184]. Though the first pathway involving PI3K-Akt-AS160-Rab GTPase leading to GLUT4 vesicle translocation is well-studied, the second insulin-signaling arm involving PI3K-Rac1 and actin remodeling to GLUT4 vesicle mobilization in skeletal muscle remains incompletely characterized.

Rac1 is a small Rho family GTPase which is typically involved in the regulation of cytoskeletal reorganization and vesicular traffic in most cell types [185]. Growing evidence supports the involvement of Rac1 in insulin-induced GLUT4 vesicle translocation to the cell surface, both in cultured L6 muscle cells and mature

primary skeletal muscle [186]. Knockdown or inhibition of Rac1 activity ablates GLUT4 translocation and subsequent glucose uptake [176]. Rac1 mediates this process by inducing cortical F-actin remodeling, which involves the recruitment of actin regulatory proteins such as cofilin and Arp2/3 to the actin filaments in skeletal muscle cells [121]. Additionally, Rac1 signals to p21-activated kinase 1 (PAK1) in skeletal muscle and facilitates its phosphorylation in response to insulin [187]. Insulin-stimulated PAK1 activation was decreased in human skeletal muscle of both acute (intralipid infusion) and chronic (obesity and Type 2 diabetes) insulin resistant states, implicating PAK1 as a required element in maintaining euglycemia and insulin sensitivity. However, the connections between PAK1, Rac1 and cortical F-actin remodeling to mediate insulin-dependent GLUT4 movement to the cell surface have yet to be established.

Several studies in mice depleted of PAK1 (whole-body $PAK1^{-/-}$ knockout) support the requirement for PAK1 in the maintenance of glucose homeostasis *in vivo*, linked to its actions in islets and skeletal muscle [132]. Although PAK1 function in islets appears to be linked to Cdc42 [188, 189], its role in skeletal muscle is predicted to be linked instead to Rac1, given that Rac1 but not Cdc42 has been shown to participate in glucose uptake. However, given the reported requirements for PAK1 in heart tissue, two cell types of the islet (α- and β-cells) and intestinal cells [190], the metabolic derangement of the PAK1 KO mouse and the role of PAK1 in skeletal muscle has remained unresolved. In addition, PAK1 serves two roles, one as a kinase in signaling actions, and another as a scaffolding protein [162]. Moreover, it is unknown whether PAK1's signaling or

scaffolding functions, or both, are required for GLUT4 vesicle translocation and glucose uptake in skeletal muscle for the regulation of whole-body glucose levels.

In the current study we tested the hypothesis that PAK1 signaling is required for insulin-stimulated GLUT4 vesicle translocation to the cell surface by contributing to cortical actin remodeling in skeletal muscle cells. Towards this end, we employed an inhibitor of PAK signaling, IPA3, to distinguish PAK1's requirement for signaling from that of scaffolding. IPA3 is a selective inhibitor of the group I PAK isoforms; another PAK inhibitor, PF-3758309, acts on both group I and II PAKs. Skeletal muscle and L6-GLUT4myc clonal muscle cells were used, as both showed identical expression of the Group 1 PAK isoforms, of which only the PAK1 isoform was found to be of significance to insulin-stimulated GLUT4 vesicle translocation. Inhibition of PAK1 signaling function completely ablated insulin-dependent GLUT4 translocation and glucose uptake in L6-GLUT4myc muscle cells. Mechanistically, IPA3 blunted the insulin-induced dynamic rearrangement of cortical F-actin in L6-GLUT4myc myoblasts. We further demonstrate that PAK1 signals to dephosphorylate cofilin, in a manner independent of LIM kinase, revealing an unusual signaling axis for actin remodeling in skeletal muscle cells.

2.3. MATERIALS AND METHODS

2.3.1. Materials

Rat L6 GLUT4-myc skeletal muscle cells expressing c-myc tagged GLUT4 protein were developed as described. MEMα medium was purchased from Invitrogen (Carlsbad, CA). Porcine insulin, rabbit anti-Actin antibody and 2-Deoxyglucose were purchased from Sigma-Aldrich (St. Louis, MO). The LifeAct-GFP plasmid was kindly provided by Dr. Louis Philipson (University of Chicago, IL [191]), and the GFP-hPID and GFP-hPID-L107F plasmids were a gift from Dr. Jonathan Chernoff (Fox Chase Cancer Center, PA [192]). An inhibitor of the activation of group I PAKs (IPA3), phospho-PAK1^{T423} and mouse anti-p-cofilin antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Phospho-PAK1^{T423}/phospho-PAK2^{T402}, PAK1, PAK2, PAK3, cofilin, phospho-ERK1/2$^{T202/Y204}$ and ERK1/2 antibodies were obtained from Cell Signaling (Danvers, MA). Fetal bovine serum and goat anti-mouse horseradish peroxidase secondary antibody were obtained from Thermo-Fisher Scientific (Rockford, IL). Goat anti-rabbit horseradish peroxidase secondary antibody was purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagent (ECL), ECL prime and SuperSignal™ Femto were purchased from GE Healthcare (Piscataway, NJ) and Pierce (Rockford, IL), respectively.

2.3.2. Cell culture

L6-GLUT4myc myoblasts were grown as monolayers in MEM-α medium supplemented with 10% fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution. L6-GLUT4-myc myoblasts at 40% confluency were differentiated into

myotubes by incubation in MEM-α medium containing 2% fetal bovine serum. For all the studies involving IPA3, L6-GLUT4-myc myoblasts (~ 90% confluency) were pre-incubated in serum-free medium for 3 hr with IPA3 added for the times indicated in the figures just prior to insulin stimulation (100 nM). Cells were harvested in 1% NP-40 lysis buffer containing 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, 137 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml leupeptin and cleared of insoluble material by centrifugation at 13,000 x g for 10 min at 4^0C. Supernatant was used for immunoblot analyses. Cells were transfected with plasmid DNA using Effectene transfection reagent (Qiagen, Valencia, CA), Lipofectamine 2000 (LifeTechnologies, Grand Island, NY) or with siRNA oligonucleotides using Jet Prime transfection reagent according to the manufacturer's protocol (Polyplus transfection, NY, USA) as recently described [193]. siRNA oligonucleotide sequences used: siPAK2 sense 5'-ggucugucaucgacccuautt-3' and antisense 5'-auagggucgaugacagacctt-3'; siControl sense 5'-uaaggcuaugaagagauactt-3' and antisense 5'-guaucucuucauagccuuatt-3', obtained from Qiagen.

2.3.3. RNA isolation and qRT-PCR

RNA was isolated from islets using the RNAeasy Fibrous Tissue Mini kit (Qiagen, Valencia, CA) and reverse-transcribed to cDNA using the Superscript First strand synthesis system (Invitrogen, Carlsbad, CA). PCR was performed using Biomix red for 30 cycles: 94°C for 1 min, 56°C for 1 min, and 71°C for 1 min, with a final

10-min elongation at 71°C and PCR products were visualized on 2% agarose gel. Primers used for the detection of PAK1 (forward: 5-tgtctgagaccccagcagta and reverse: 5'-cccgagttggagtaacagga), PAK2 (forward 5-aacaccagcactgaacacca and reverse 5'-cttggcaccactgtcaacat) PAK3 (forward 5-gcagcacatcagtcgaatacca and reverse 5'-tttatttggtgcagctggt) and GAPDH (5′-atggtgaaggtcggtgtgaacg and reverse 5′-gttgtcatggatgaccttggcc) were obtained from IDT (Coralville, IA). The qRT-PCR reaction was performed using CFX Connect Real-Time system (Bio-Rad, Hercules, CA) and amplifications were done using the Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA). The thermal cycling conditions for the reaction were as follows: 50°C for 2-min hold (UDG incubation), 95°C for 2-min hold, 40 cycles of 95°C for 15 s, and 60°C for 30 s. PCR products were visualized on 2% agarose gels. Relative quantification in gene expression levels were quantified using the $2^{-\Delta Ct}$ method where relative mRNA levels of PAK1, 2 and 3 reported are normalized to GAPDH.

2.3.4. Live-cell imaging

L6-GLUT4myc myoblasts were seeded on MatTek glass bottom culture dishes at a density of 300,000 cells per 35 mm dish. At ~40% confluency cells were transfected with LifeAct-GFP plasmid using Effectene transfection reagent (Qiagen, Valencia, CA). Live-cell imaging was performed on cells 48 hr post-transfection. Briefly, on the day of the experiment the cells were pre-incubated in serum-free KRPH buffer (120 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, and 2 mM $CaCl_2$) supplemented with 5 mM D-glucose for 3 h, then IPA3 or vehicle (DMSO) added for 50 min. LifeAct-GFP imaging

was performed on a custom spinning-disk confocal microscope with a heated 60x Plan Apo Lambda 1.4 NA objective lens and sample chamber with temperature, humidity and CO2 regulation built around a CSU-10 spinning disk confocal head (Yokogawa) which is controlled by NIS Elements AR v 4.10 (Nikon Instruments). Images were captured every 60 sec starting 1 min before the addition of insulin and continued through until 10 min after the addition of insulin. Movies of each condition are shown as Supplemental data movies 1-4.

2.3.5. Cell surface GLUT4myc detection

Cell surface GLUT4myc detection was performed as described earlier [194]. Briefly, L6-GLUT4-myc myoblasts or myotubes were pre-incubated in serum-free medium containing IPA3 (25 μM) or vehicle (DMSO) for 40 min followed by insulin stimulation (100 nM for 20 min), all at 37^{o}C. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT), blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at RT and incubated with mouse anti-Myc antibody overnight at 4^{o}C. Cells were extensively washed with PBS and then incubated with infrared (IR)-conjugated secondary antibody for 1 h at RT. Immunofluorescence intensity of the IR-conjugated secondary antibody was quantified using the LiCor infrared imaging system (LI-COR Biosciences, Lincoln, NE) and data normalized to SYTO 60 (Invitrogen, Carlsbad, CA) , a red fluorescent nucleic acid stain.

2.3.6. 2-Deoxyglucose uptake assay

The 2-deoxyglucose uptake assay was performed as described [195]. Briefly, L6 myotubes were pre-incubated in serum-free FCB buffer (125 mM NaCl, 5 mM

KCl, 1.8 mM $CaCl_2$, 2.6 mM $MgSO_4$, 25 mM HEPES, 2 mM pyruvate, 2% BSA) for 30 min, IPA3 or vehicle added for 40 min, and then stimulated with insulin for 20 min. Glucose uptake was initiated by the addition of 2-deoxy[1,2,-^{3}H] glucose (Perkin Elmer, Waltham, MA) and uptake terminated after 5 min by four quick washes with ice cold PBS followed by addition of 250 µl of 1N NaOH for quantitation of [^{3}H] using liquid scintillation. Data were normalized for variability in protein concentration, as determined by Bradford assay.

2.3.7. Immunoblotting

Proteins in cell lysates were resolved using 10-12% SDS-PAGE and transferred to PVDF membranes for immunoblotting. Immunoreactive bands were visualized using ECL, ECL Prime, or Supersignal Femto reagents and imaged using a BioRad Chemi-Doc gel documentation system. Phosphorylated and total ERK1/2 proteins were visualized using goat anti-mouse 680 and anti-rabbit 800 simultaneously for LiCor imaging.

2.3.8. Statistical analysis

All data were expressed as the average ± SE using Students t-test. Time course and dose response data were evaluated with one-way ANOVA and a Tukey post-hoc test using GraphPad Prism™ (La Jolla, CA).

2.4. RESULTS

2.4.1. PAK expression and inhibition by IPA3 in skeletal muscle cells

To determine if the defects in insulin-stimulated GLUT4 accumulation at the cell surface in the PAK1 KO mice were related to PAK1 signaling, we employed IPA3, an allosteric inhibitor of PAK kinase activation and signaling activity that is selectively initiated by the small GTPases Rac1 and Cdc42 [196, 197]. IPA3 binds covalently to the PAK1 regulatory domain and prevents binding to these upstream activators, although pre-activated PAK is not inhibited by IPA3. IPA3 is capable of inhibiting all PAK isoforms of the Group 1 family (includes PAKs 1-3), therefore the expression of all three of these isoforms in skeletal muscle was evaluated using immunoblotting and quantitative real-time PCR (qRT-PCR). Of the three existing isoforms, only PAK1 and PAK2 were found in skeletal muscle of C57BL6 mice (Fig. 1A-B), and this pattern was recapitulated by L6-GLUT4-myc myoblasts. PAK1 and PAK2 migrate at distinct molecular weights on SDS-PAGE (68 and 61 kDa, respectively), and immunoblotting using a phospho-specific PAK1/2$^{T423/402}$ antibody showed that PAK1 displays substantial insulin-stimulated phosphorylation/activation (versus basal=1.0, insulin-stimulated=2.0 ± 0.3, $p<0.05$) within 10 min (Fig. 1C). In fewer than half of all experiments using this antibody, PAK2 showed low levels of phosphorylation. By contrast, PAK1 consistently displayed a high level of activation in response to insulin, further confirmed using a PAK1^{T423} selective phospho-specific antibody in Fig. 1D (versus basal=1.0, insulin-stimulated=1.6 ± 0.1, $p<0.05$).

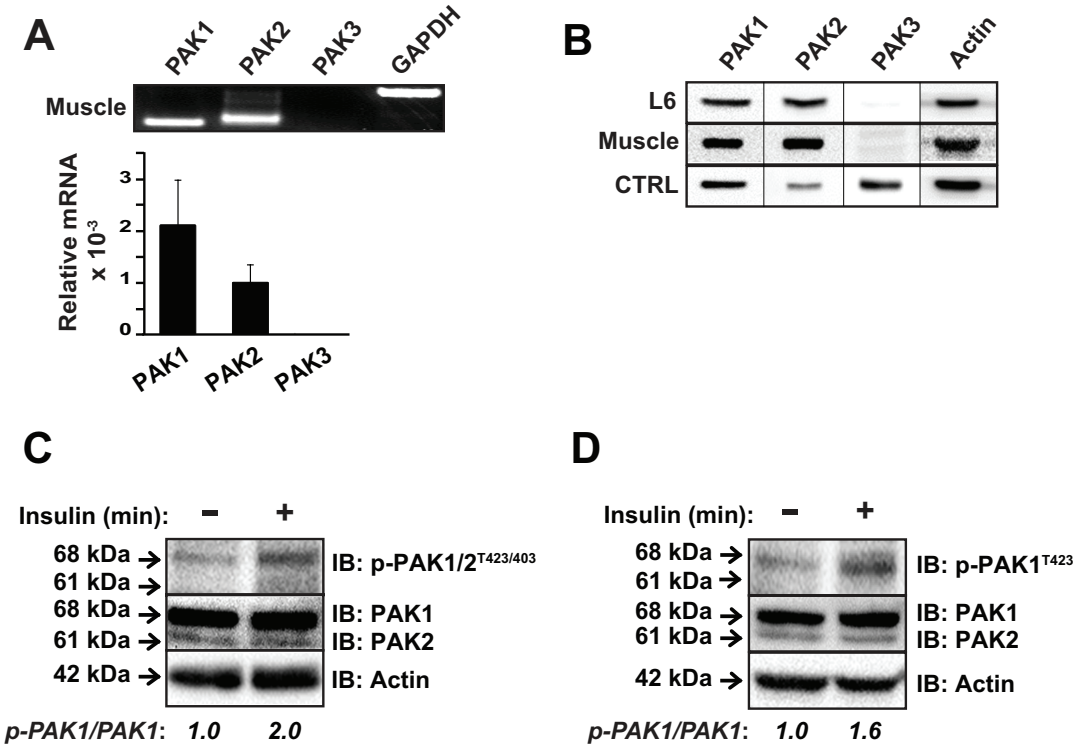


Figure 2.1. Group I PAK isoforms in skeletal muscle and L6-GLUT4-myc cells. Hindlimb muscle from wild-type (WT) mice was homogenized and analyzed for the three Group I PAK members PAK1, 2 and 3 for ***A)*** mRNA content using qRT-PCR (normalized to GAPDH) from three sets of tissues, and ***B)*** protein expression. L6-GLUT4-myc myoblasts were assessed similarly for protein expression of all three Group I PAKs; mouse brain lysates served as control (CTRL) as they co-express all three isoforms. Vertical black lines denote splicing of lanes from the PAK1, PAK2, PAK3 and actin immunoblots. ***C)*** Lysate proteins from L6-GLUT4-myc myoblast cells left unstimulated or insulin-stimulated (100 nM, 10 min) were resolved by 12% SDS-PAGE for immunoblot detection of phosphorylated- and total- PAK1 and PAK2 proteins and ***D)*** specifically for phosphorylated PAK1. Data are representative of three independent experiments, with average insulin-stimulated p-PAK1/total PAK1 ratios shown below the blots, normalized to basal=1.0 for each experiment.

We next optimized the dosage and time-dependence of IPA3 action in myoblasts, based upon prior experimentation using IPA3. IPA3 provoked a >60% inhibition of insulin-stimulated PAK1 phosphorylation at 25 and 30 μM (Fig. 2A). At 25 μM, inhibition of PAK1 activation was consistently achieved within 60 min (Fig. 2B). No PAK2 phosphorylation was detected in any of these experiments. IPA3 treatment at the most effective time and dose was without impact upon insulin-dependent Akt phosphorylation (Fig. 2C), reminiscent to the lack of impact on Akt phosphorylation displayed by skeletal muscle of PAK1 KO mice. Therefore, 25 μM IPA3 treatment for 60 min was used in subsequent experiments.

2.4.2. Insulin-stimulated PAK1 phosphorylation is essential for GLUT4 vesicle translocation and glucose uptake into skeletal muscle cells

We next examined the effect of IPA3 on insulin-stimulated GLUT4 vesicle exocytosis and glucose uptake. Insulin stimulated a 170% increase in GLUT4 at the cell surface in myoblasts treated with vehicle alone (DMSO), and IPA3 treatment abrogated this effect of insulin (Fig. 3A). IPA3 did not exert a negative impact upon basal/unstimulated levels of GLUT4 (versus vehicle control=1.0, IPA3=1.1 ± 0.1, $p>0.05$). Glucose uptake was measured in myotubes, since at this stage the contribution of the housekeeping glucose transporter GLUT1 to glucose uptake is minimal. Consistent with the reduction in GLUT4 translocation, IPA3 treatment markedly reduced insulin-stimulated glucose uptake into L6 myotubes (Fig. 3B). In another approach, myoblasts were transfected to express

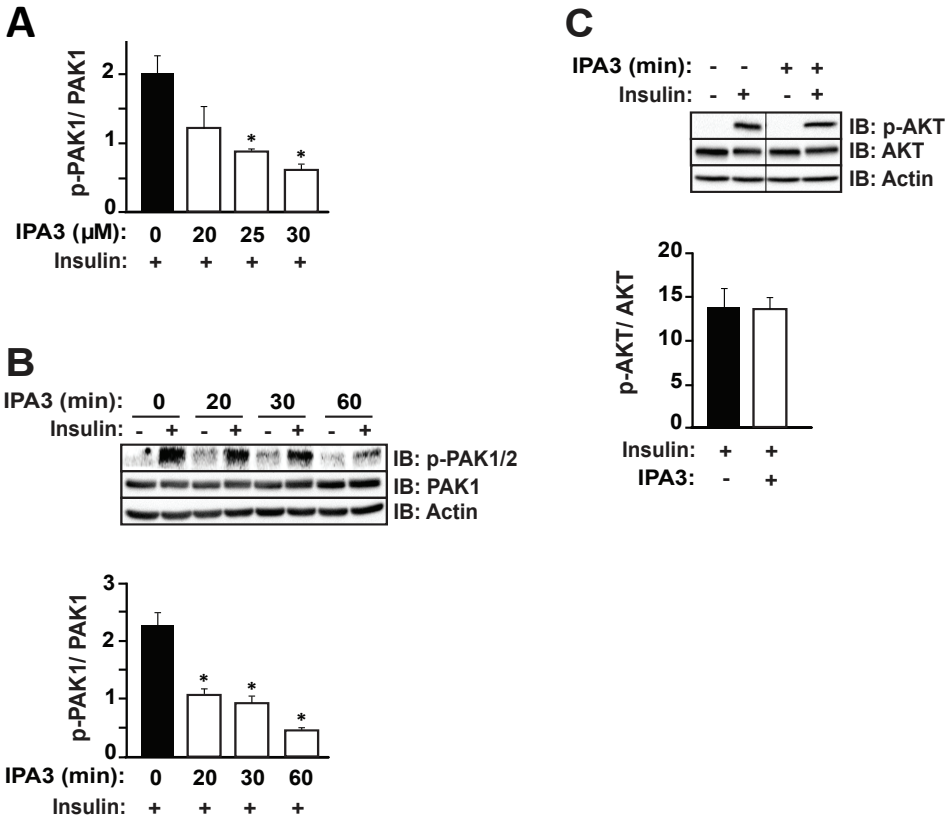


Figure 2.2. IPA3 inhibits insulin-stimulated PAK1 phosphorylation in L6-GLUT4-myc myoblasts. ***A)*** Dose-optimization: L6 myoblasts were pretreated with IPA3 at 20, 25 or 30 μM for 10 min followed by insulin stimulation for an additional 10 min. Detergent cleared cell lysates were prepared and proteins resolved by SDS-PAGE for immunoblot and optical density scanning quantitation for p-PAK1 relative to total PAK1 (in arbitrary units). ***B)*** Time course: L6 myoblasts were treated with 25 μM IPA3 for 0, 20, 40 or 60 min, with insulin added in the final 10 min (100 nM). Cell lysates were immunoblotted for p-PAK1 and PAK1 and quantified as in A) above. ***(C)*** p-AKT and total AKT were immunoblotted and quantified from cells treated with vehicle (-) or 25 μM IPA3 for 60 min as described in A) above. Bar graphs represent the average ± SE of three independent cell passages; *p<0.05. The vertical black line denotes splicing of lanes from within the same gel exposure.

the PAK1 auto-inhibitory domain (PID), a known dominant-negative for PAK1 signaling actions. Indeed, PID-expressing myoblasts showed attenuated insulin-stimulated GLUT4 translocation to the cell surface, relative to that of cells expressing the non-inhibitory mutant PID-L107F (Fig. 3C). PID had no negative impact upon basal levels of surface GLUT4 (versus L107F control=1.0, PID=1.2 ± 0.1, $p>0.05$). Since IPA3 also has the capacity to inhibit PAK2, we tested the effect of PAK2 depletion in L6 myoblasts. Transfection with siPAK2 oligonucleotides achieved 75% PAK2 knockdown relative to non-targeting control oligonucleotides (Fig. 3D), yet this was without effect upon insulin-stimulated GLUT4 translocation (Fig. 3E). Combined with evidence showing skeletal muscle PAK1 KO to exhibit attenuated insulin-stimulated GLUT4 translocation [198], these data suggest that PAK1 signaling is required for insulin-stimulated GLUT4 vesicle exocytosis in L6 myoblasts, revealing a selective importance for PAK1 in this process.

2.4.3. PAK1 signaling is required for insulin-induced F-actin remodeling in skeletal myoblasts

Since PAK1 inhibition by IPA3 did not affect Akt phosphorylation, we tested the possibility that PAK1 contributes to the insulin-signaling arm involving PI3K-Rac1 and actin remodeling. Actin remodeling is a prerequisite for GLUT4 vesicle translocation and glucose uptake into L6 myoblasts [199] and mature skeletal muscle [114]. PAK1 is known to impact actin remodeling in other cell types,

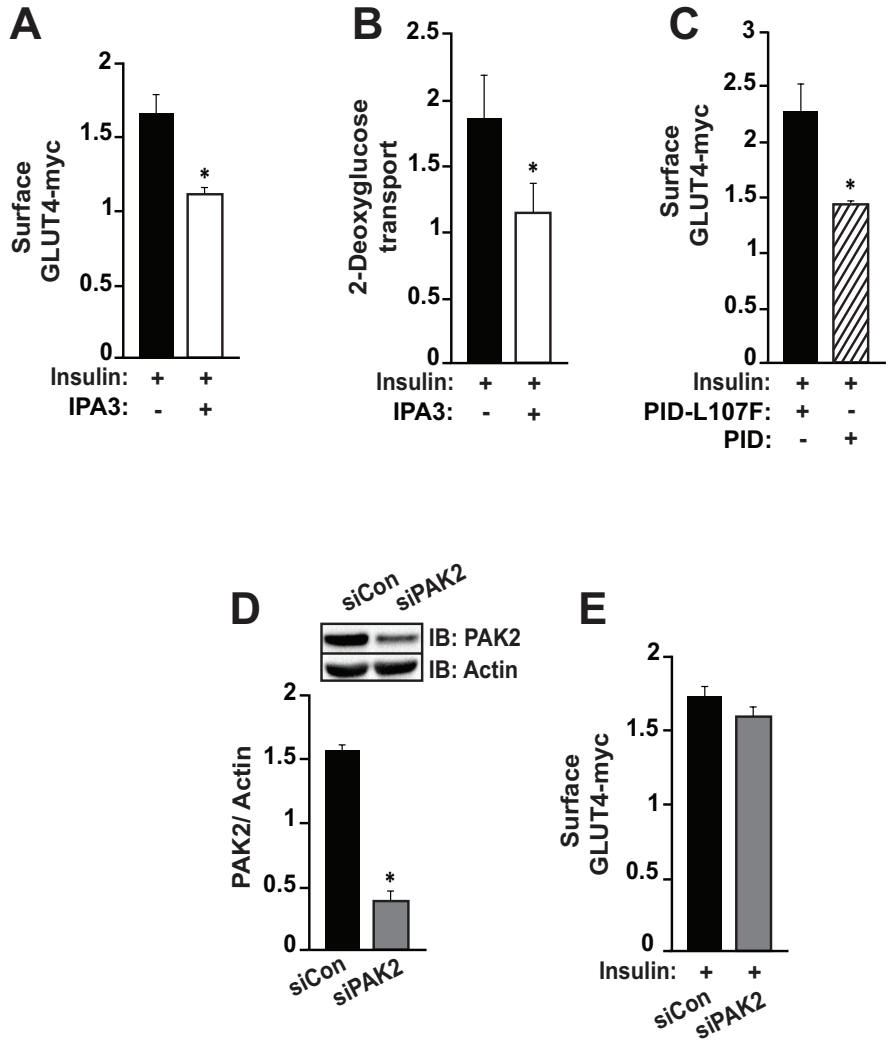


Figure 2.3. PAK1 activity is essential for insulin stimulated GLUT4 vesicle translocation and glucose uptake in skeletal muscle cells. ***A)*** Surface GLUT4: L6-GLUT4-myc myoblasts treated with IPA3 or vehicle (DMSO) were stimulated with insulin (100 nM, 20 min) for LiCor analyses of surface GLUT4 levels. Immunofluorescent intensity of cell surface GLUT4 was normalized to nucleic acid staining dye, Syto 60, and data displayed as the fold-stimulation of insulin-stimulated surface GLUT4, relative to unstimulated/basal level. Bars represent the average ± SE of four independent cell passages; *p<0.05, vs vehicle (-) treated cells. ***B)*** Glucose uptake: L6-GLUT4-myc myotubes were used for 2-deoxyglucose uptake assays as described in *Methods*. Data represent the average (± SE) fold stimulation in response to insulin relative to basal level glucose uptake, in at least three independent passages of cells; *p<0.05, vs vehicle (-) treated cells. ***C)*** L6-GLUT4-myc myoblasts were transfected to express GFP-tagged PAK1 inhibitory domain (PID) or non-inhibitory control (PID-

L107F) and assessed for fold stimulation of insulin-stimulated GLUT4 as described in A) above. Bars represent the average ± SE of three independent cell passages; *p<0.05, vs PID-L107F-expressing cells. ***D)*** Myoblasts were transfected with control (siCon) or PAK2-selective (siPAK2) siRNA oligonucleotides, normalized to actin (arbitrary units). Bars represent the average ± SE of three independent cell passages; *p<0.05, vs siCon. ***E)*** GLUT4 vesicle translocation assays from siPAK2 or control (siCon) transfected cells, as described in A) above. No significant differences were detected in four independent experiments. Experiment for Figure 2C was done by our collaborator Tim T. Chiu.

either by inducing F-actin formation/assembly or reducing F-actin in a cell type-specific manner. All studies to date have used fixed-cell imaging to study this process, with the inherent caveat that basal and insulin-stimulated images derive from different cells. Instead, we sought to capture the real-time changes in actin polymerization in single cells using live-cell imaging of L6 myoblasts harboring the LifeAct-GFP biosensor. LifeAct is a 17 residue peptide from the actin binding protein Abp140 linked to the N-terminus of GFP to form LifeAct-GFP, which has been shown to bind specifically to F-actin in live cells without adversely affecting F-actin dynamics. L6 myoblasts transfected to express LifeAct-GFP exhibited F-actin remodeling within minutes of insulin stimulation, as can be viewed in real-time movies (Supplemental movies 1-4), with still images captured at the end of the 10 min period (Fig. 4); remodeling is visualized as membrane ruffling (see regions denoted by arrows). Notably, IPA3 treatment completely blocked all insulin-induced F-actin remodeling across the entire imaging period in all independent movies captured. These experiments are the first to demonstrate the time-lapse events of insulin-stimulated actin remodeling in skeletal muscle cells, wherein PAK1 plays an essential role in the process.

2.4.4. Insulin-stimulated PAK1 signaling in L6 myoblasts

We next questioned whether PAK1 signaling to evoke F-actin remodeling occurred via a canonical or non-canonical route. The canonical pathway from PAK1 to evoke cofilin phosphorylation is through activation of LIM kinase (LIMK1/2) in other cell types. Incongruent with this, LIMK knockdown in L6

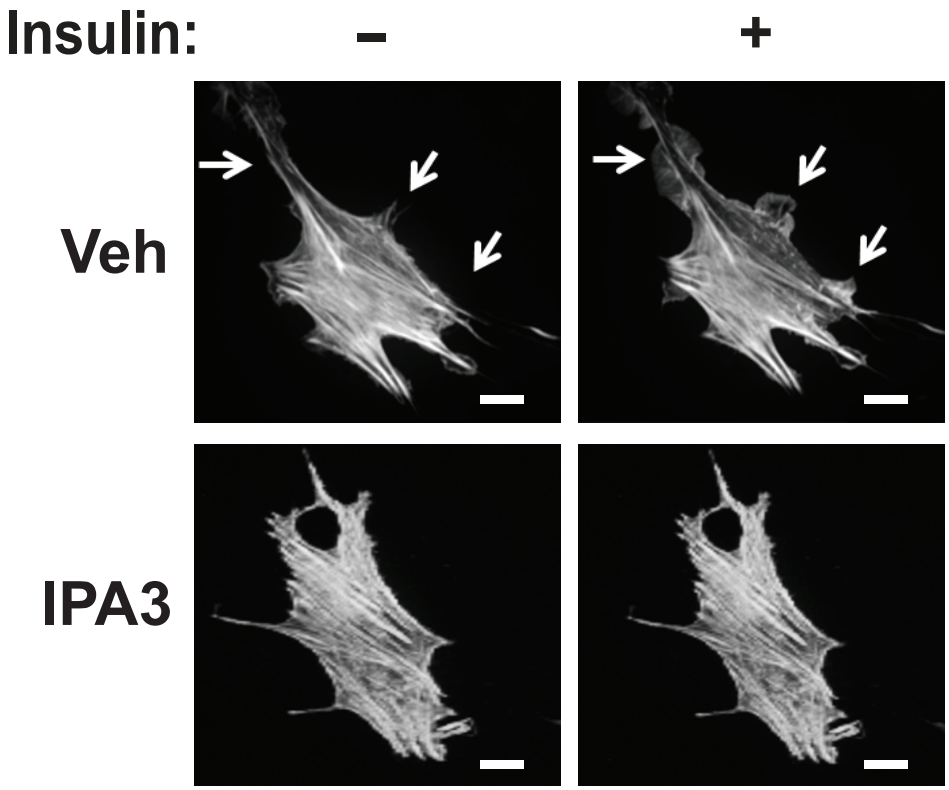


Figure 2.4. Insulin-induced PAK1 signaling is important for insulin-induced F-actin remodeling in L6-GLUT4-myc myoblasts. L6-GLUT4-myc myoblasts were transfected to express the LifeAct-GFP biosensor for live-cell imaging of F-actin remodeling. Myoblasts treated with vehicle or IPA3 were imaged using an custom spinning-disk confocal microscope and captured every min from 1 min prior insulin addition and on through to 10 min after insulin stimulation (100 nM). Arrows denote regions of ruffling. Images represent still images taken from at least four cell movies of each treatment condition conducted using four independent passages of cells.

myoblasts failed to substantially affect cofilin phosphorylation. Hence, IPA3 was used to evaluate this canonical pathway, serving as an alternative to depleting PAK1 which otherwise also impedes its scaffolding function. Insulin stimulation triggered a > 2-fold increase in p-PAK1 activation, and this was inhibited by IPA3 (Fig. 5A); IPA3 was without impact upon basal activation (versus vehicle=1.0, IPA3= 1.0 ± 0.2, $p>0.05$), as seen in Fig. 2B. In the same cell lysates, insulin stimulated LIMK phosphorylation by ~2-fold; however, IPA3 failed to inhibit this activation (Fig. 5B). IPA3-treated lysates showed slightly higher pLIMK levels (versus basal vehicle of 0.5 ± 0.03, IPA3 = 1.0 ± 0.07), although since insulin-stimulated pLIMK were similarly altered, the fold activation level was comparable to that of vehicle-treated cells. Issues with pLIMK and LIMK antibodies required that dual gels be used, one for LIMK and the other for pLIMK immunoblotting, with each normalized for deviations in protein loading to actin, and then the pLIMK/total LIMK calculated. The pLIMK antibody recognized multiple non-specific bands, and the true pLIMK bands were discerned by paired migration against the correct band in stimulated HUVEC cells as recommended by the manufacturer. As shown previously, and opposite to the canonical pathway, insulin stimulation decreased p-cofilin levels to ~50% of that from unstimulated cells. Importantly, IPA3-treated myoblasts showed blunted insulin-stimulated cofilin dephosphorylation (Fig. 5C). Consistent with its lack of effect upon surface GLUT4 or glucose uptake, IPA3 was without effect upon basal levels of cofilin phosphorylation (versus control=1.0, IPA3=0.9 ± 0.1, $p>0.05$). Insulin-induced phosphorylation of ERK served as an unrelated pathway control for the

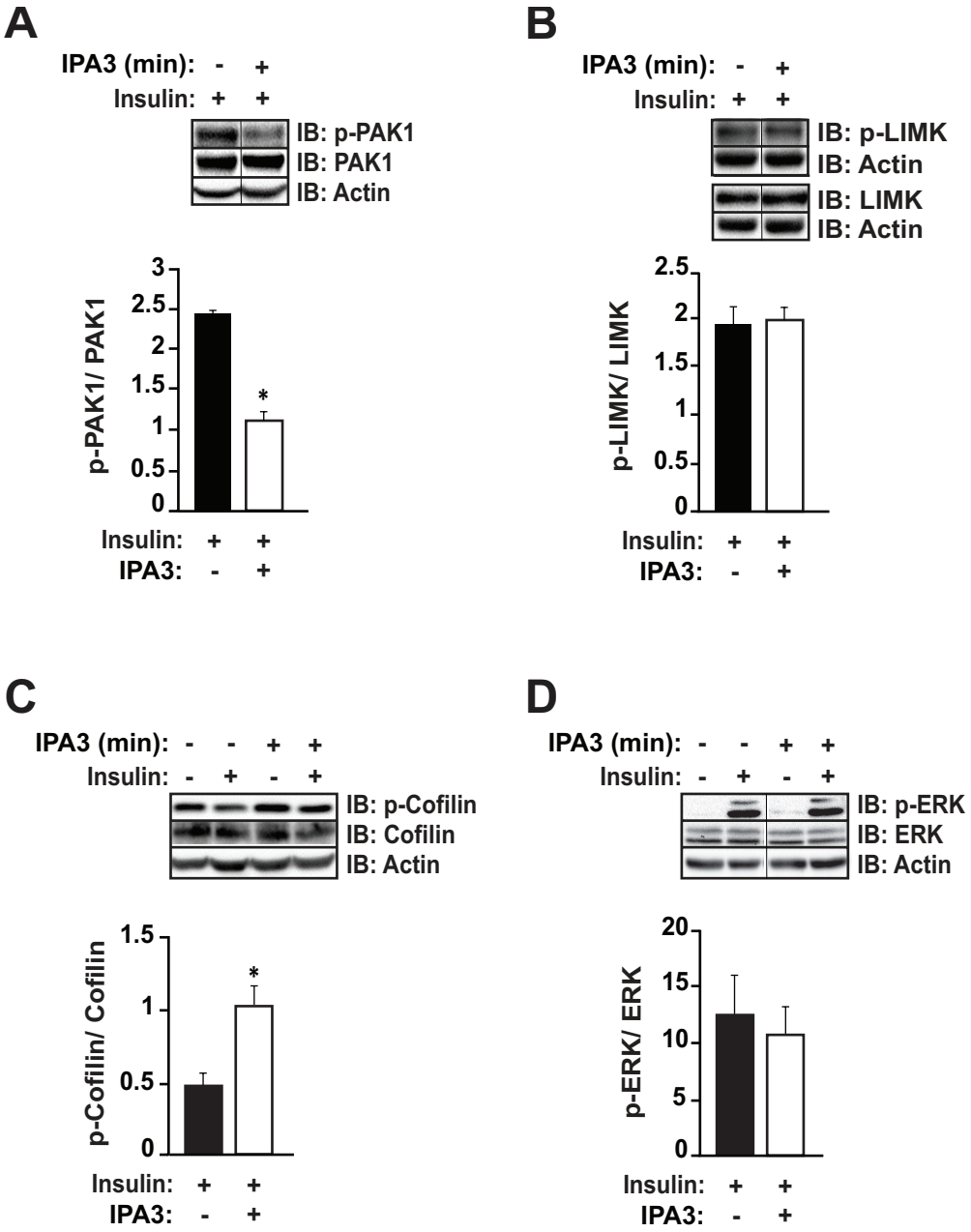


Figure 2.5. PAK1 signaling is required selectively for insulin-stimulated cofilin de-phosphorylation in L6-GLUT4-myc myoblasts. L6-GLUT4-myc myoblasts treated with vehicle or IPA3 were stimulated with insulin (100 nM, 10 min) and resulting detergent cleared cell lysates resolved on 12% SDS-PAGE for simultaneous immunoblot detection of ***A)*** p-PAK1 and total PAK1, ***B)*** p-LIMK and total LIMK ***C)*** p-cofilin and total cofilin, and ***D)*** p-ERK and total ERK. Optical density scanning was used for quantification, with bar graphs representing the average ± SE of fold changes in activations evaluated in four independent experiments; *p<0.05. Vertical black lines denote splicing of lanes from within the same gel exposure within each figure panel.

PAK-specific effects of IPA3, and to demonstrate that the cells were appropriately responsive to insulin (Fig. 5D). Taken together, these findings highlight the importance of PAK1 signaling for cofilin de-phosphorylation, which proceeds through a non-canonical, LIMK-independent pathway.

2.5. DISCUSSION

PAK1 KO mice exhibit peripheral insulin resistance [132]. However, since PAK1 is known to function both as a scaffolding protein as well as a signaling protein, its ablation in the PAK1 KO mouse model left elucidation of its specific function(s) in regulating insulin sensitivity unresolved. Moreover, this whole body knockout model of PAK1 precluded assigning its mode of action directly to skeletal muscle. Here, we show that insulin-stimulated PAK1 activation is an integral step in the signaling cascade that enables actin remodeling and GLUT4 vesicle translocation to stimulate glucose uptake into skeletal muscle cells. Showing for the first time the kinetics of insulin-stimulated actin remodeling in live skeletal muscle cells by time-lapse imaging using the F-actin biosensor LifeAct-GFP, we reveal that PAK1 activation is required to display this actin remodeling. Since IPA3-mediated PAK1 inhibition failed to impair Akt phosphorylation but did impair actin remodeling, PAK1 signaling is likely required in the Akt-independent PI3K→Rac1 signaling cascade of insulin action. Prior work shows that insulin-stimulated cofilin dephosphorylation is required for GLUT4 translocation [121]; our data fully recapitulate this work. Mechanistically, our data expand upon this to show that IPA3-mediated inhibition of p-PAK1 abrogates the dephosphorylation of cofilin, a finding incongruent with the canonical, stimulus-induced p-PAK1→p-

LIMK→p-cofilin response. Moreover, cofilin dephosphorylation occurred in a manner independent of LIMK, suggesting that PAK1 contributes to actin remodeling via a non-canonical pathway.

The requirement for PAK1 signaling to evoke the dephosphorylation of cofilin is unusual, only otherwise reported to occur in the MCF-7 cells [200]. PAK1 signals to numerous substrates in a variety of cell types [201], one of which is LIMK, which in turn leads to the phosphorylation (inactivation) of cofilin to regulate the actin cytoskeletal dynamics. In contrast, insulin-stimulated actin remodeling in skeletal muscle cells involves cofilin *de*phosphorylation. The model drawn from these findings proposes that GLUT4 vesicle translocation and fusion requires active cycling or 'remodeling' of actin. Our live-cell imaging demonstrates this dynamic cycling to occur in response to insulin, and that PAK1 signaling is required to facilitate the cycling. Supporting this concept in the context of cofilin dephosphorylation by insulin, Slingshot1 (SSH1) was identified as the cofilin phosphatase in the L6 muscle cells. SSH1 activity was shown to predominate over that of LIMK in these muscle cells, with LIMK knockdown having little impact upon cofilin phosphorylation status. Our data support this and further show that while PAK1 is upstream of cofilin, IPA3-mediated inhibition of PAK1 fails to inhibit LIMK activation. Alternative to activation by PAK1, LIMK may become activated in response to insulin stimulation via Rho kinase or 'ROCK' activation [202]. Regardless though, this LIMK pathway is expected to be a lesser contributor to actin remodeling given the substantial impact of IPA3-mediated PAK1 inhibition. An additional open question is whether SSH1 activity is regulated by PAK1,

either directly or indirectly. SSH1 is activated upon its dephosphorylation, in response to calcineurin as well as to formation of polymerized actin; SSH1 contains an actin binding domain. SSH1 is inactivated by protein kinase D1, phosphorylating a serine residue located in its actin-binding motif. The PAK4 isoform, a Group II PAK, is known to negatively regulate SSH1 activity, although this is the opposite of what one would expect given that in skeletal muscle cells, cofilin is dephosphorylated in response to insulin and in a pPAK1-dependent manner. Hence, our data would appear to be consistent with PAK1 acting upstream as an indirect regulator of SSH1, via its ability to induce actin polymerization.

In L6 skeletal muscle cells, cofilin dephosphorylation requires a prior accumulation of polymerized actin driven by Arp2/3. The Arp2/3 complex, an actin-nucleating complex in association with cofilin, is implicated in the regulation of cortical actin remodeling in skeletal muscle cells. In other cell types PAK1 is known to regulate actin remodeling by phosphorylating p41-ARC, a regulatory subunit of Arp2/3 complex [122]. Additionally, the Arp2/3 complex with its activator N-WASP is involved in the recycling of GLUT4 transporters via regulating cortical actin remodeling in adipocytes. Studies are underway to investigate the role and placement of p41-ARC and N-WASP in the PAK1-dependent insulin signaling pathway in skeletal muscle cells. Additionally, PAK1 signaling to other downstream factors such as cortactin, Myo1c and Filamin A should be investigated, since these targets are known to bind to PAK1 in non-

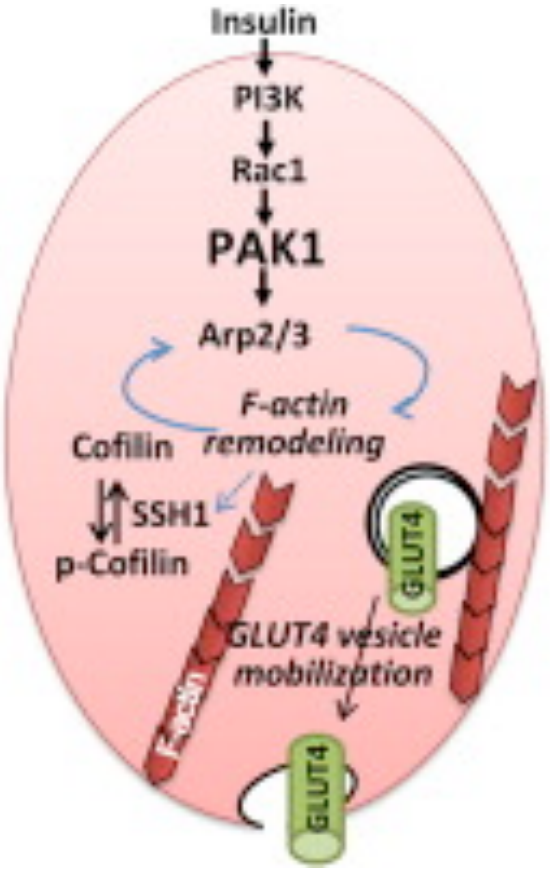


Figure 2.6. Proposed model for insulin-stimulated PAK1 signaling in skeletal muscle. Insulin-stimulated PAK1 activation initiates F-actin remodeling by regulating the activity of cofilin and is required for mobilization of GLUT4 vesicles to the cell surface, resulting in glucose uptake.

muscle cells and have been implicated as indirect effectors of glucose uptake [203]. In L6-GLUT4-myc myotubes the depletion of Rac1 results in the ablation of PAK1 activation, as does the treatment of mouse skeletal muscle with the Rac1 inhibitor NSC23766. Moreover, skeletal muscle-specific Rac1 KO mice have impaired skeletal muscle PAK1 signaling, suggesting that PAK1 is downstream of Rac1. However, PAK1 has also been implied to function upstream of Rac1 in other cell types, by phosphorylating RhoGDI to yield activated Rac1 [204]. Hence, future studies are required to investigate the potential additional placement of PAK1 upstream of Rac1 using PAK1 inhibitors and skeletal muscle-specific PAK1 KO mice.

The fact that IPA3 action on GLUT4 translocation and cofilin phosphorylation status is reminiscent to that of PAK1 depletion in vivo suggests that its actions on muscle cells are unlikely to arise from effects on targets other than PAK1. However, we cannot discount that the inhibitory action on actin remodeling is entirely due to its action on PAK1.

In summary, we have demonstrated that PAK1 signals to cofilin in response to insulin and facilitates the cortical actin remodeling in skeletal muscle cells. With this, PAK1 mediates the GLUT4 vesicle translocation to the cell surface and results in glucose uptake into the skeletal muscle cells. Interestingly, a GWAS study has localized PAK1 to the T2D susceptibility locus on human Chromosome 11 [205, 206], and PAK1 deficiency is linked to glucose intolerance, insulin resistance and T2D in human and rodent models of these pathophysiological states [207]. Altogether, these data suggest that a decrease in PAK1

abundance/function might be a potential risk factor for pre-diabetes susceptibility. Hence strategies/methods to restore the function or abundance of PAK1 in skeletal muscle could be useful for re-establishing insulin action and euglycemia.

CHAPTER 3

PAK1 SIGNALS TO p41-ARC AND N-WASP IN RESPONSE TO INSULIN TO EVOKE ACTIN REMODELING AND GLUCOSE UPTAKE IN SKELETAL MUSCLE CELLS

3.1. SYNOPSIS

Defects in GLUT4 translocation are associated with peripheral insulin resistance, pre-clinical diabetes and eventual progression to T2D. Recruitment of GLUT4 to the PM of skeletal muscle cells requires filamentous (F)-actin remodeling. Although in vitro data implicates the neural Wiskott-Aldrich syndrome protein (N-WASP) in insulin-dependent cortical F-actin rearrangement, the mechanism of action involving N-WASP, and any relatedness to skeletal muscle function, remains elusive. Here we show that inactivation of N-WASP by its specific inhibitor, Wiskostatin, fully abrogates the insulin-stimulated increase in GLUT4 translocation to the plasma membrane in skeletal muscle cells. Toward interrogating the underlying mechanism, interactions between N-WASP and actin as well as actin binding proteins Cortactin and ARP2/3 complex were assessed, given that the latter proteins are implicated in actin remodeling in clonal muscle cells. Indeed, a ~1.5 fold increased binding of Cortactin to N-WASP with insulin stimulation was detected using mouse skeletal muscle lysates. Moreover, interaction of N-WASP with both actin and p41-ARC (a regulatory subunit of the ARP2/3 complex) also increased upon insulin stimulation, and this interaction was abrogated upon inhibition of PAK activation by IPA3 (an inhibitor of PAK activation) in skeletal muscle cells. In sum, these results suggest that N-WASP facilitates Cortactin- and p41-ARC-mediated F-actin remodeling for insulin-stimulated GLUT4 vesicle translocation to the PM of skeletal muscle cells.

3.2. INTRODUCTION

Insulin stimulates glucose uptake into skeletal muscle and adipose tissue by mobilizing intracellular vesicles containing the glucose transporter GLUT4 to the PM [179],[180]. Integration of GLUT4 into the PM facilitates glucose entry into the cell. Skeletal muscle accounts for ~80% of insulin-dependent postprandial glucose uptake [208]; defective glucose clearance results in insulin resistance and contributes to the development of T2D. Hence, targeting mechanisms to promote insulin-stimulated GLUT4 vesicle mobilization provides a means to restore insulin sensitivity.

Skeletal muscle cells respond to insulin via the presence of insulin receptors at the cell surface, triggering the canonical insulin signaling pathway: insulin binds to the insulin receptor to induce autophosphorylation, which in turn induces the tyrosine phosphorylation of insulin receptor substrate (IRS), then PI3K. In skeletal muscle, the pathway downstream of PI3K bifurcates into two parallel and independent arms: one arm leads to phosphorylation of AKT, and the second arm leads to the activation of the monomeric small GTPase protein Rac1 [95]. Rac1 activation evokes the phosphorylation of its effector protein, PAK1. While the events downstream of the AKT arm of insulin-signaling are well-documented in these glucose uptake mechanisms, the Rac1-PAK1 aspect remains undetermined.

In humans, insulin-induced PAK1 activation is impaired in skeletal muscle in acute (intralipid infusion) and chronic (high fat diet-induced obesity and T2D) insulin resistant states, implicating the requirement of PAK1 for insulin-dependent

glucose uptake/disposal [176]. Studies using whole-body PAK1 knockout mice replicate these findings, as did use of the Group 1 PAK1 inhibitor IPA3, providing evidence for PAK1 involvement in actin remodeling to promote GLUT4 vesicle translocation [209]. Stimulus-induced actin cytoskeletal remodeling requires the action of multiple actin binding proteins that control the spatial and temporal polymerization and depolymerization events. In a non-canonical signaling mechanism, PAK1 was found to regulate insulin-induced actin remodeling in skeletal muscle cells in a LIM Kinase independent manner, signaling to cofilin (actin severing protein) to initiate actin depolymerization. However, the mode by which PAK1 regulates actin polymerization has yet to be established.

Studies of actin remodeling in skeletal muscle cells have revealed Cortactin to be required for insulin-induced GLUT4 translocation and glucose uptake [123](Nazari H etal 2011). Cortactin is an actin nucleation-promoting factor that mediates the assembly and organization of actin cytoskeletal meshwork [210], and has been shown in other cell types to be phosphorylated by PAK1. In Hep2b clonal hepatocytes, the phosphorylation of Cortactin by PAK1 specifically at Ser405 and Ser418 increased the affinity of Cortactin for a known enhancer of actin polymerization, neuronal Wiskott-Aldrich syndrome protein (N-WASP) [124]. However, N-WASP has not been investigated in the process of insulin-induced actin remodeling or glucose uptake in skeletal muscle cells. Moreover, again in other cell types, PAK1 is known to regulate actin remodeling by phosphorylating p41-ARC, a regulatory subunit of the ARP2/3 complex[122]. Although ARP2/3 has been implicated as a positive factor in GLUT4 translocation in skeletal

muscle cells, involvement of p41-ARC remains untested. Hence, PAK1 may signal through p41-ARC-ARP2/3 or cortactin-N-WASP, or both pathways, to evoke actin remodeling and glucose uptake via GLUT4 vesicle mobilization in skeletal muscle cells.

Towards testing these possible pathways, we used chemical inhibitors, live cell imaging and immunoprecipitation studies to discern the insulin-induced changes in protein-protein complexes concurrent with changes in glucose uptake and GLUT4 translocation. N-WASP was determined to be essential as a downstream mediator of PAK1 in insulin-induced actin remodeling, GLUT4 translocation, and associated with Cortactin. Further, PAK1 activation was required for its association with p41-ARC and actin in skeletal muscle cells. Altogether, these findings elucidate new elements of the insulin signaling pathway downstream of Rac1-PAK1 in skeletal muscle that dictate the cortical F-actin remodeling and subsequent mobilization of GLUT4 vesicles and fusion to the cell surface.

3.3. MATERIALS AND METHODS

3.3.1. Materials

Rat L6 GLUT4-myc skeletal muscle cells expressing c-myc tagged GLUT4 protein were purchased from Kerafast and developed as described [211]. MEM α medium was purchased from Invitrogen (Carlsbad, CA). Porcine insulin, DMSO, wiskostatin (WISK) and rabbit anti-Actin antibody were purchased from Sigma (St.Louis, MO). EnzyFluo glucose uptake assay kit was obtained from BioAssay systems (Hayward, CA). The LifeAct-GFP plasmid was kindly provided by Dr.

Louis Philipson (University of Chicago, IL [191]). The F-G actin Ratio kit was obtained from Cytoskeleton (Denver, CO). IPA3, protein G+ beads and anti c-myc antibody were purchased from Santa Cruz Biotech (Santa Cruz, CA). Phospho-PAK1^{T423}/phospho-PAK2^{T402}, PAK1, N-WASP, Cortactin and antibodies were obtained from Cell Signaling (Danvers, MA). p41-ARC antibody was from Abcam (Company). Fetal bovine serum and goat anti-mouse horseradish peroxidase secondary antibody were obtained from Thermo-Fisher Scientific (Rockford, IL). Goat anti-rabbit horseradish peroxidase secondary antibody was purchased from Bio-Rad (Hercules, CA). Enhanced chemiluminescence reagent (ECL), ECL prime and SuperSignal™ Femto were purchased from GE Healthcare (Piscataway, NJ) and Pierce (Rockford, IL), respectively.

3.3.2. Cell culture

L6-GLUT4myc myoblasts were grown as monolayers in MEM-α medium supplemented with 10% fetal bovine serum and 1% (v/v) antibiotic-antimycotic solution. L6-GLUT4-myc myoblasts at 40% confluency were differentiated into myotubes by incubation in MEM-α medium containing 2% fetal bovine serum [212, 213]. For all the studies involving IPA3, L6-GLUT4-myc myoblasts (~ 90% confluency) were pre-incubated in serum-free medium for 2 hr with IPA3 added for the times indicated in the figures just prior to insulin stimulation (100 nM). Similarly, all the experiments using WISK were pre-incubated in serum-free medium for 2 hr, with WISK and insulin (100 nM) added simultaneously. Cells were harvested in 1% NP-40 lysis buffer containing 25 mM HEPES, pH 7.4, 1% Nonidet P-40, 10% glycerol, 50 mM sodium fluoride, 10 mM sodium

pyrophosphate, 137 mM NaCl, 1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml aprotinin, 1 µg/ml pepstatin, 5 µg/ml leupeptin and cleared of insoluble material by centrifugation at 13,000 x g for 10 min at 4^0C. The cleared lysate was used for immunoblot analyses.

3.3.3. Cell surface GLUT4myc detection

Cell surface GLUT4myc detection was performed as described [194]. Briefly, L6-GLUT4-myc myoblasts were incubated in serum-free medium containing WISK (10 µM) or vehicle (DMSO) for 20 min together with insulin (100 nM) at 37^{o}C. Cells were then fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT), blocked in Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE) for 1 h at RT and incubated with mouse anti-Myc antibody (1:100) overnight at 4^{o}C. Cells were extensively washed with PBS and then incubated with infrared (IR)-conjugated secondary antibody for 1 h at RT. Immunofluorescence intensity of the IR-conjugated secondary antibody was quantified using the LiCor Odyssey CLx infrared imaging system (LI-COR Biosciences, Lincoln, NE) and data normalized to SYTO 60 (Invitrogen, Carlsbad, CA), a red fluorescent nucleic acid stain.

3.3.4. Immunoblotting

Proteins in cell lysates were resolved using 10% SDS-PAGE and transferred to PVDF or Nitrocellulose membranes for immunoblotting. Immunoreactive bands were visualized using Enhanced Chemiluminescence (ECL) or ECL Prime reagents and imaged using a Chemi-Doc Touch gel documentation system (Bio-

Rad). N-WASP and Cortactin proteins were visualized using goat anti-rabbit 680 secondary antibody (1:10000) for detection using Odyssey-CLx LI-COR.

3.3.5. Live-cell imaging

L6-GLUT4myc myoblasts were seeded on MatTek glass bottom culture dishes at a density of 300,000 cells per 35 mm dish. At ~40% confluency, cells were transfected with the LifeAct-GFP plasmid using Effectene transfection reagent (Qiagen, Valencia, CA). Live-cell imaging was performed on cells 48 hr post-transfection. Briefly, on the day of the experiment the cells were pre-incubated in serum-free KRPH buffer (120 mM NaCl, 2.5 mM KCl, 20 mM HEPES, 1.2 mM $MgSO_4$, 1 mM NaH_2PO_4, and 2 mM $CaCl_2$) supplemented with 5 mM D-glucose for 3 h, then DMSO or WISK was added 10 min before the addition of 100 nM insulin for live-cell imaging. LifeAct-GFP imaging was performed on a Zeiss Axio Observer with a Plan-Apochromat 63x objective and sample chamber with temperature, humidity and CO_2. Images were captured every 60 sec starting 1 min before the addition of insulin and continued through until 12 min after the addition of insulin.

3.3.6. Immunoprecipitation

L6-GLUT4myc myoblasts were incubated in serum-free medium for 2 hr, pre-treated with IPA3 for 50 min and stimulated with 100 nM insulin for 20 min. Initially, 2.5 mg of whole cell lysate was pre-cleared by incubating with 0.5 µg of c-myc antibody and 20 µl of Protein G Agarose beads for 30 min. Proteins were immunoprecipitated by incubating the pre-cleared lysate with 3.75 µg of N-WASP antibody overnight, followed by incubation with 80 µl of Protein-G Agarose beads

for 2 hr. Immunoprecipitated proteins were washed three times with lysis buffer and eluted with SDS sampling buffer containing DTT. Eluted proteins were resolved using 10% SDS-PAGE and transferred to PVDF or nitrocellulose membranes for immunoblotting. Ponceau S staining was used to evaluate protein loading. Since N-WASP antibodies failed for immunoblot detection of N-WASP immunoprecipitated, possibly due to steric hindrance of the epitope, cells were also transfected with recombinant GFP-tagged N-WASP and anti-GFP co-immunoprecipitations performed.

3.3.7. Statistical analysis

All data were expressed as the average ± SE using Students t-test. Time course and dose response data were evaluated with one-way ANOVA and a Tukey post-hoc test using GraphPad Prism™ (La Jolla, CA).

3.4. RESULTS

3.4.1. N-WASP is required for insulin-stimulated GLUT4 vesicle translocation to support glucose uptake.

To determine the role of N-WASP in insulin-dependent GLUT4 translocation and glucose uptake in skeletal muscle cells, WISK, a specific inhibitor of N-WASP was used. WISK is an allosteric inhibitor of N-WASP that interacts with a cleft in the regulatory GTPase binding domain of N-WASP and stabilizes the native auto-inhibited conformation. Multiple dosages of WISK were tested in myoblasts to determine if any effects upon insulin-induced GLUT4 translocation to the PM were apparent. Insulin induced the mobilization of GLUT4 from the intracellular compartments to the plasma membrane of L6-GLUT4myc myoblasts, and as little as 10 μM WISK attenuated insulin-stimulated GLUT4 mobilization by 80% (Fig. 3-1a-b). Higher concentrations of WISK exhibited aberrant effects on basal GLUT4 transport to the PM. Therefore, WISK was used at 10 μM for 20 min in subsequent experiments.

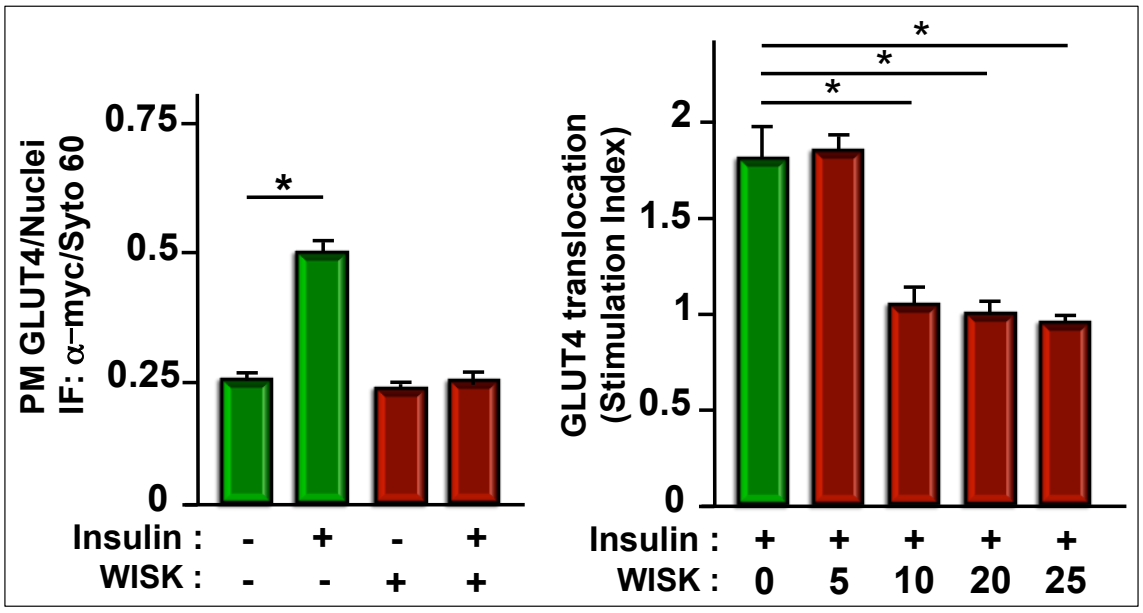


Figure 3.1. Inhibition of N-WASP blunts insulin-dependent GLUT4 translocation in L6 GLUT4myc myoblasts. A) L6-GLUT4myc myoblasts were treated with DMSO- or 10 μM Wiskostatin(WISK) and stimulated with 100 nM insulin for 20 min simultaneously. Cells were fixed and left unpermeabilized for labeling with anti-myc antibody. Immunofluorescent intensity of cell surface GLUT4 was normalized to the nucleic acid staining dye, Syto 60. Values are means ± SEM. N=3, *p<0.05 B) L6-GLUT4myc myoblasts were treated with 0, 5, 10, 20, and 25uM Wiskostatin together with 100 nM Insulin for a total of 20 mins. Cells were fixed and left unpermeabilized for labeling with anti-myc antibody. Immunofluorescent intensity of cell surface GLUT4 was normalized to the nucleic acid staining dye, Syto 60. Values are means ± SEM. N=3, *p<0.05

3.4.2. N-WASP regulates insulin-induced localized F-actin remodeling in L6-GLUT4myc myoblasts.

To determine the requirement for N-WASP signaling in the process of F-actin remodeling in skeletal muscle cells, live-cell imaging of L6 myoblasts harboring the Lifeact-GFP biosensor was performed, as described previously [209]. LifeAct is a 17 residue peptide from the actin binding protein Abp140 linked to the N-terminus of GFP to form LifeAct-GFP, binding specifically to F-actin in live cells without adversely affecting F-actin dynamics. Insulin-stimulated changes in actin polymerization in single cells of L6 myoblasts were captured over a period of 10 min, showing actin remodeling within 5-6 min of insulin addition (Fig. 3-2a). The addition of 10 μM WISK at the time of insulin addition fully ablated any actin remodeling.

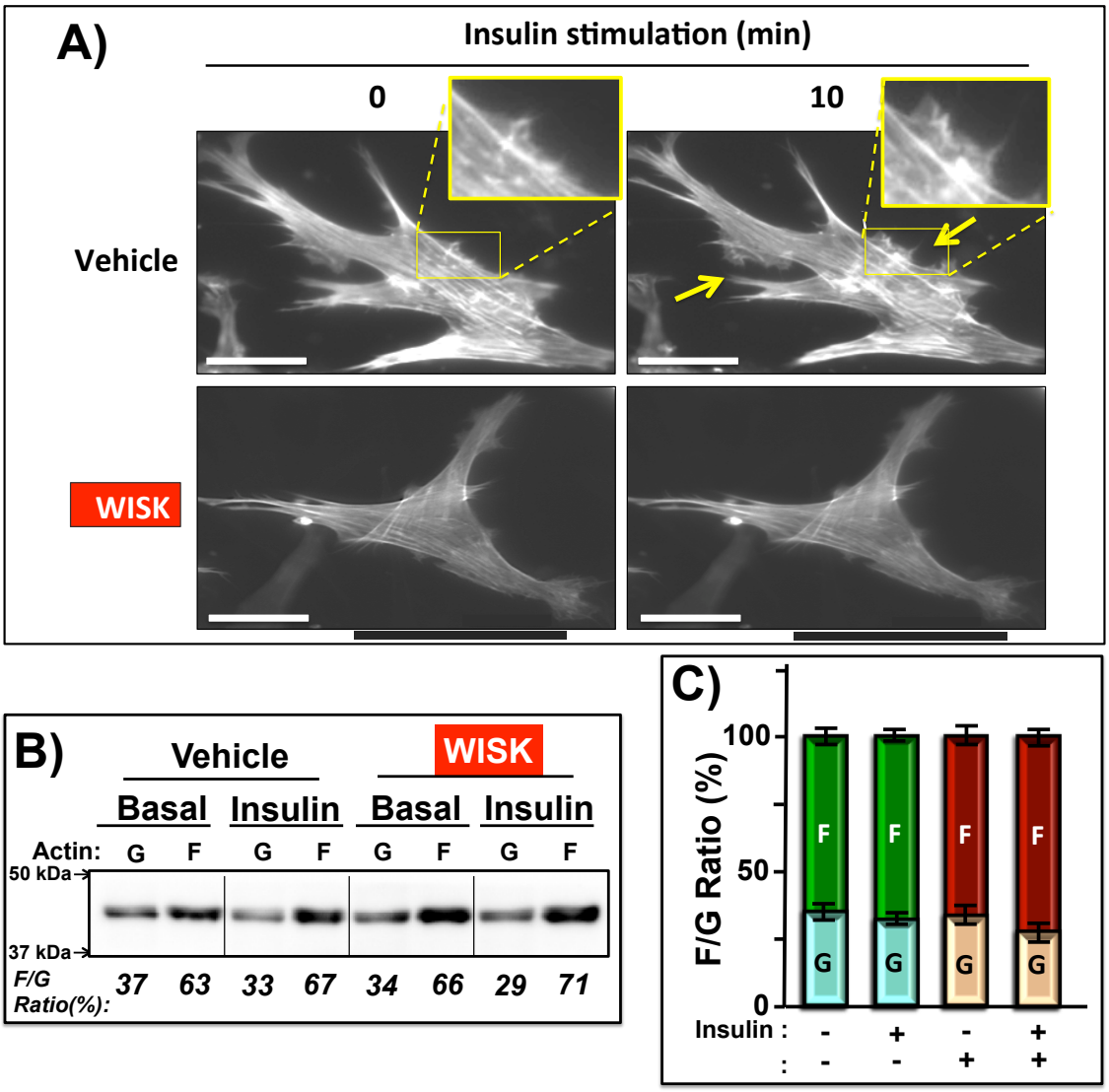


Figure 3.2. Insulin-stimulated F-actin remodeling requires N-WASP activation. A) L6-GLUT4myc myoblasts were transfected with Lifeact-GFP. After 48 h, cells were pretreated with vehicle (DMSO) or 10 μM Wiskostatin for 10 min and live cell confocal imaging was initiated. F-actin remodeling was monitored every minute from 1 min prior to insulin addition to 10 min after insulin addition. Arrows indicate sites of F-actin remodeling. At least 20 GFP-positive cells were live-imaged, with >10 treated with WISK, from 3 independent passages of L6 cells. B) Filamentous (F) and Globular (G) actin from L6-GLUT4myc myoblasts treated with vehicle (DMSO) or 10 μM WISK, left unstimulated (Basal) or stimulated with 100 nM insulin for 20 min, were resolved on SDS-PAGE for immunoblotting using anti-actin. A representative blot showing F/G-actin ratio is calculated as a percent of total per sample. C) Quantitative bar graph representation of F/G actin ratio from three independent passages of L6 cells; $p>0.05$ for all comparisons.

WISK did not alter global actin cytoskeletal changes, since the F/G-actin ratio remained similar, as compared with vehicle-treated cells (Fig. 3-2b-c). Interestingly, insulin did not evoke global changes to F/G-actin ratios either, supporting the prevailing concept in the field that insulin induces localized actin remodeling. These data suggest that N-WASP is required for insulin stimulated actin remodeling to support GLUT4 vesicle translocation in skeletal muscle cells.

3.4.3. N-WASP requires PAK1 activity for its interaction with actin and Cortactin in response to insulin.

Although PAK1 is known to regulate insulin-induced GLUT4 translocation via F-actin remodeling in skeletal muscle cells, the downstream signaling pathway involving PAK1 remains unexplored. Previously, we have shown that PAK1 indirectly regulates Cofilin activity to induce actin depolymerization, which is one of the two arms of actin remodeling. Here, we explored the downstream signaling cascade of PAK1 that regulates actin polymerization, the other arm of actin remodeling. Immunoprecipitation studies with N-WASP revealed a 6-fold increase in association of actin with N-WASP in response to insulin stimulation (Fig. 3-3a), and this interaction was significantly reduced by ~67% in cells treated with the PAK inhibitor, IPA3. The actions of insulin and IPA3 were validated in each independent experiment using a phospho-specific PAK antibody (Fig. 3-3b), Similar to WISK inhibition, inhibition

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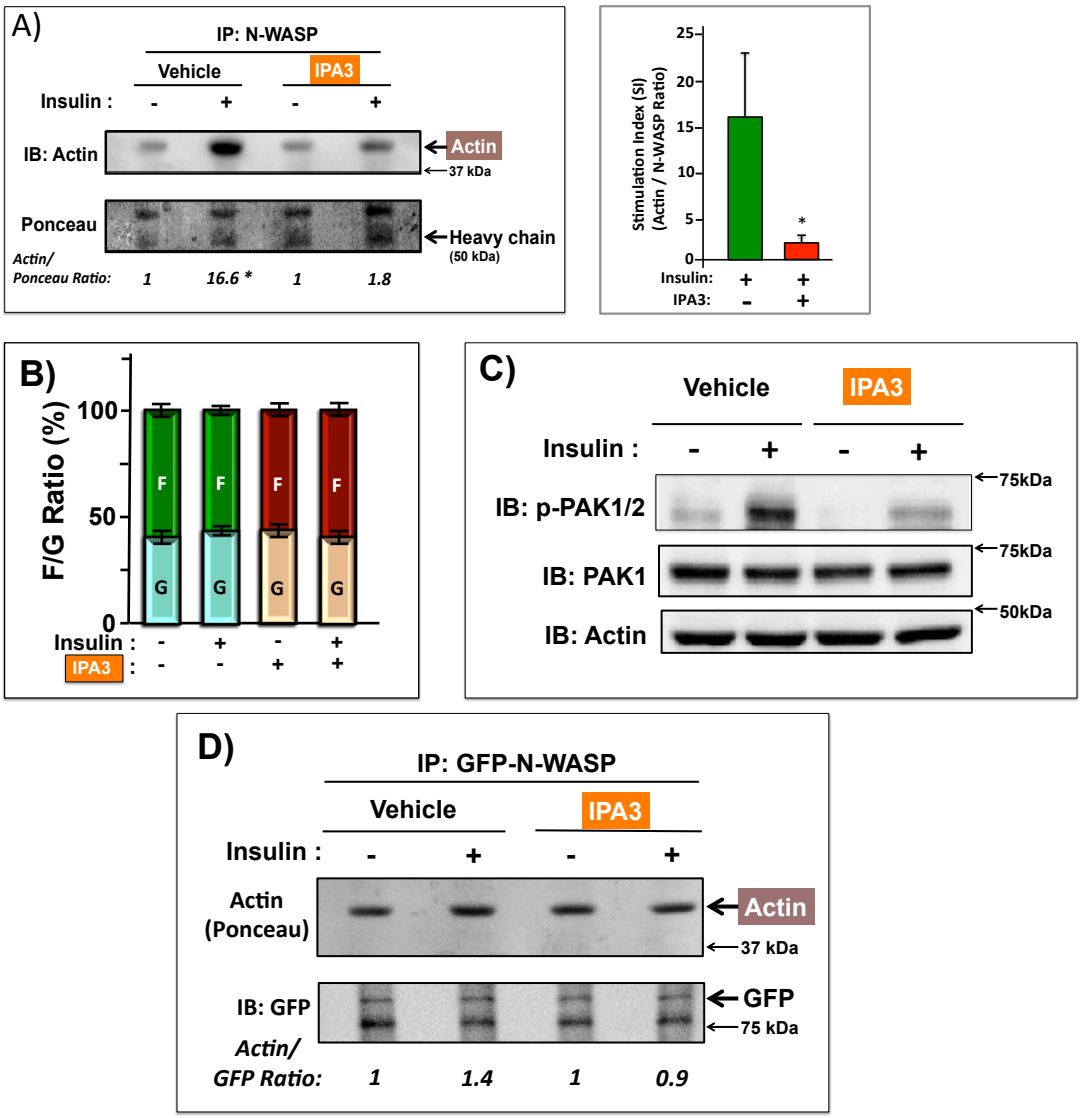


Figure 3.3. Inhibition of PAK1 activation decreases the insulin-stimulated association of Actin with N-WASP in L6 GLUT4myc myoblasts. A) L6-GLUT4myc myoblasts were pre-treated with DMSO- or 25 μM IPA3 for 50 min and stimulated with 100 nM insulin for additional 10 min. Whole cell lysates were subjected to immunoprecipitation with anti-N-WASP antibody and precipitated proteins were resolved using SDS-PAGE for immunoblot analyses. Actin levels were normalized for loading using ponceau S staining of the same blot, in three independent sets of cell lysates. Values represent the means ± SEM; *p<0.05, vs. insulin-stimulated vehicle control. B) L6-GLUT4myc myoblasts pretreated with vehicle (DMSO) or 25 μM IPA3 were stimulated with 100nM insulin for 10 min. F and G-actin were separated by ultracentrifugation and analyzed on SDS-PAGE. C) Whole cell lysates from the above were analyzed for p-PAK1/2$^{T423/T402C}$ and total PAK1 protein levels. D) IP of EGFP-N-WASP protein from L6-GLUT4myc myoblasts overexpressing EGFP-NWASP protein was performed as mentioned in (A) and Actin levels were normalized for loading to GFP. Bars represent the means ± SEM in 3 independent sets of L6 cells; p>0.05.

of PAK activation had no global effects on the F/G actin ratio (Fig. 3-3c). These data support the concept of insulin-stimulated PAK1 activation fostering the association of N-WASP with the actin cytoskeleton to evoke localized actin remodeling in skeletal muscle cells.

Cortactin is a known regulator of actin polymerization and its phosphorylation by PAK1 on Ser405 and Ser418 has been shown to increase its interaction with N-WASP in different cell types [124]. To interrogate the role of PAK1 in the interaction of N-WASP and Cortactin, hindlimb muscle homogenates prepared from wild-type C57B/L6J (WT) and whole body $PAK1^{-/-}$ homozygous knockout (KO) mice injected with insulin or saline were subjected to immunoprecipitation with anti-N-WASP antibody. Strikingly, immunoprecipitation of N-WASP resulted in a 2.6-fold increased co-precipitation of Cortactin from insulin-stimulated WT muscle homogenates, as compared with saline-injected homogenates (Fig. 3-4). The N-WASP association with cortactin was virtually undetectable in the skeletal muscle of $PAK1^{-/-}$ KO mice, under either basal or insulin-stimulated conditions. Notably, cortactin in primary mouse skeletal muscle was >90 kDa in molecular weight, higher than that in L6 cells (~75-80 kDa). Blocking peptide studies show this band to be bona fide Cortactin; mass spectrometry studies are underway to confirm this unusual finding.

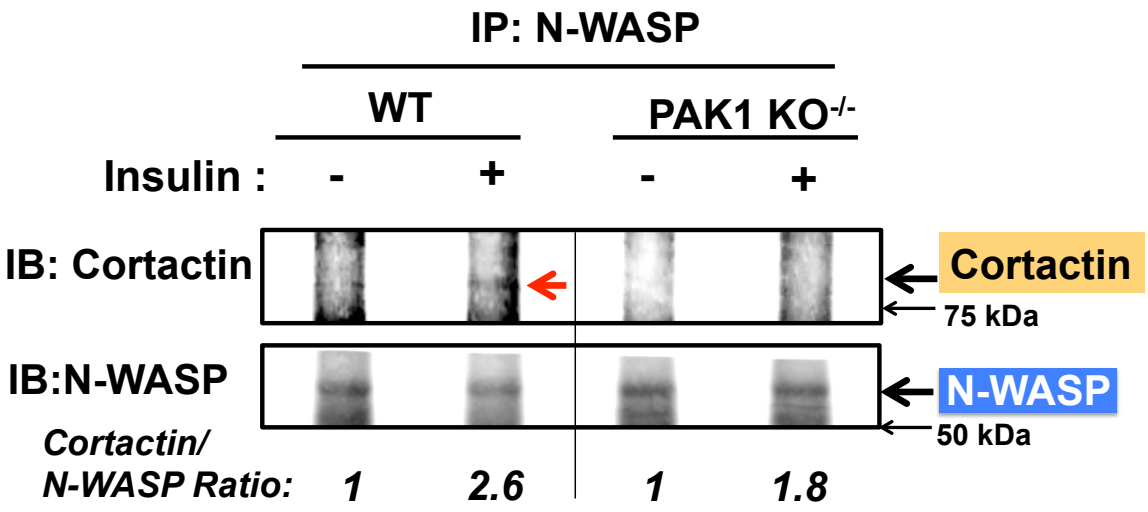
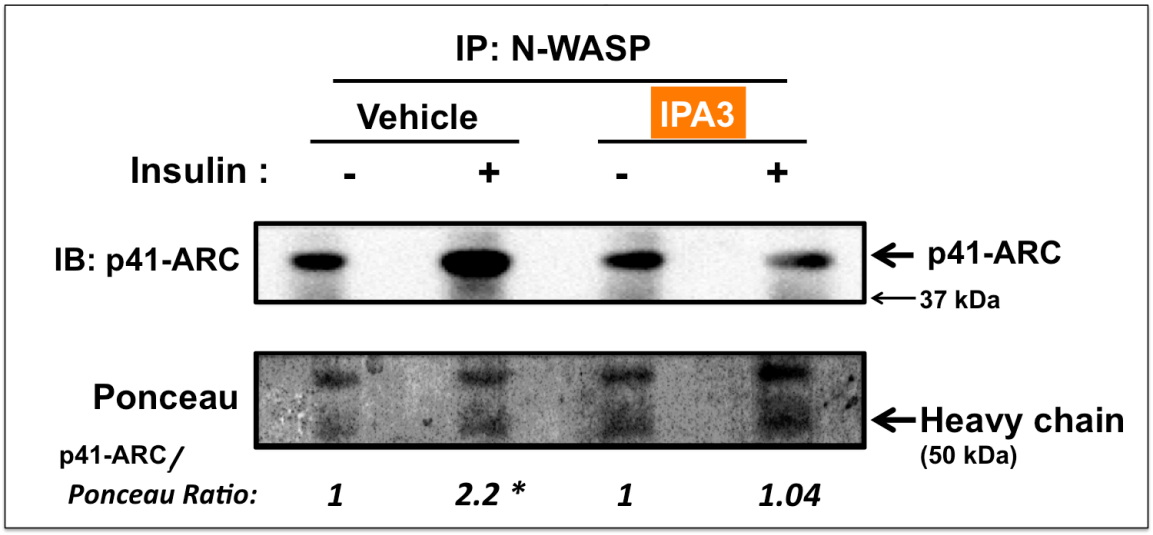


Figure 3.4. PAK1 regulates insulin-dependent N-WASP-Cortactin interaction in primary skeletal muscle tissue. Hindlimb muscle of saline/ insulin stimulated WT and global PAK1 $KO^{-/-}$ mice was homogenized and 4 mg protein of tissue homogenate was used in immunoprecipitation reactions containing anti-N-WASP antibody. Precipitated proteins were resolved on SDS-PAGE for immunoblot (IB) analysis using antibodies detecting cortactin and N-WASP. The cortactin/N-WASP ratio is derived from one full set of mouse hindlimb homogenates.

3.4.4. PAK1 activation is required for the interaction with N-WASP in insulin-stimulated L6-GLUT4myc myoblasts.

N-WASP and Cortactin are actin nucleation promoting factors that regulate actin polymerization through the actin branching complex, ARP2/3. PAK1 has been shown to regulate this protein complex by phosphorylating p41-ARC, a regulatory subunit of ARP2/3 complex, in a breast cancer cell line. Immunoprecipitation studies revealed the interaction of p41-ARC with N-WASP in L6-GLUT4myc myoblast lysates, and the abundance of these complexes increased in response to insulin-stimulation (Fig. 3-5a). This insulin-stimulated N-WASP-p41-ARC association was ablated by pre-treatment of myoblasts with the PAK inhibitor IPA3. These findings suggested that in skeletal muscle cells, PAK1 might signal downstream to p41-ARC to foster the associations of N-WASP-cortactin in an insulin-dependent manner.



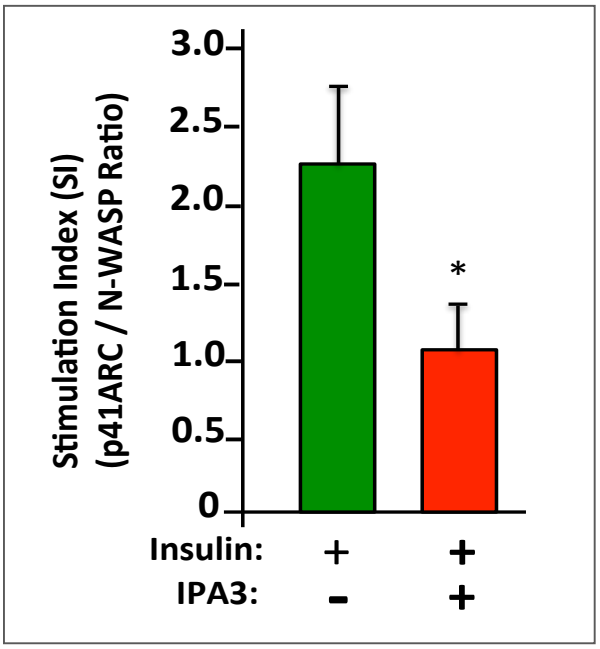


Figure 3.5. Insulin-dependent interaction of p41-ARC with actin is regulated by PAK1 in L6-GLUT4myc myoblasts. L6-GLUT4myc myoblasts were pre-treated with DMSO- or 25 μM IPA3 for 50 min and stimulated with 100 nM insulin for additional 10 min. Whole cell lysate were immunoprecipitated with N-WASP antibody and interacting partners were determined by western blotting analysis. Ratio of p41-ARC/Ponceau S staining of the same blot is listed below the ponceau S. Values are means ± SEM. N=3, *p<0.05.

3.5. DISCUSSION

In the current study, we report the existence of a new signaling pathway downstream of PAK1 that regulates cortical F-actin polymerization in skeletal muscle tissue and cells. As depicted in the model derived from our new and previously published findings (Fig. 3-6), insulin stimulation induces Rac1 activation, giving rise to PAK1 phosphorylation and p41-ARC association with N-WASP, which promotes N-WASP binding to Cortactin and F-actin polymerization. These insulin-responsive interactions were ablated by exposure of cells to pharmacological inhibitors of PAK1 phosphorylation (IPA3), placing them downstream and dependent upon PAK1 activation. Supportive of the pharmacological data, skeletal muscle from PAK1 knockout mice also lacked these interactions. PAK1 activation may foster p41-ARC-N-WASP interaction via its reported ability to directly serve as kinase to phosphorylate p41-ARC; mass spectrometry studies are currently underway to test this feature. Live-cell imaging showed that WISK signaling to support F-actin polymerization in muscle cells facilitated localized and not global action polymerization, consistent with the concept of actin remodeling, and was required for insulin-stimulated GLUT4 translocation and fusion with the plasma membrane.

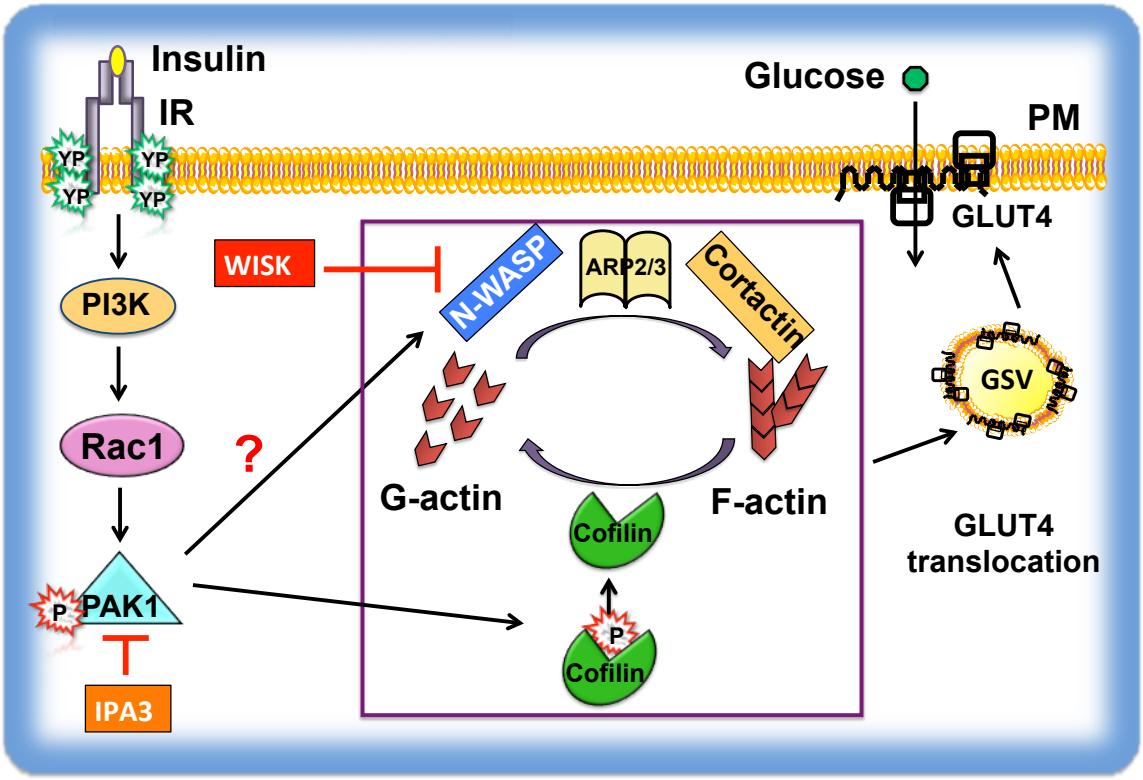


Figure 3.6. Insulin binds to insulin receptor (IR) and results in the activation of Rac1 in an phosphatidylinositol-3-kinase (PI3K) dependent manner. Activated Rac1 likely recruits its downstream effector PAK1 and initiates the dynamic cortical F-actin remodeling which is regulated by actin binding proteins, Cofilin, N-WASP, Cortactin and ARP2/3 complex. The cortical F-actin remodeling facilitates the movement of GLUT4 storage vesicles (GSV) to the cell surface and results in glucose uptake.

Finding p41-ARC to serve as a downstream effector of PAK1 signaling in the insulin-induced pathway towards actin remodeling represents the first implication of this factor in the process of insulin-stimulated GLUT4-mediated glucose uptake. PAK1 phosphorylates p41-ARC^{T21} in MCF-7 cells upon epidermal growth factor (EGF) stimulation [122]. p41-ARC is one of two principle ARP2/3 subunits used somewhat interchangeably amongst cell types, with the other being ARPC1A. Interestingly, recent studies by Abella *et al* in both *in vitro* as well as *in vivo* (in HeLa cells) demonstrated that cortactin binds and stabilizes ARPC1B/ ARPC5L containing complex to promote actin assembly and assist in the formation of long actin tails compared to the short actin tails formed by the p41-ARC/ARPC5 containing ARP2/3 complexes. However, our findings here along with others [214] show p41-ARC to be the more likely participant in skeletal muscle. Although ubiquitously expressed, p41-ARC is less-often reported as participating in these ARP2/3 complexes.

PAK1 signaling through p41-ARC to N-WASP also provides a novel aspect to the canonical insulin signaling cascade in skeletal muscle cells. N-WASP was shown to be important for GLUT4 translocation in 3T3L1 adipocytes [215], but signaling to evoke actin remodeling is distinct from that in skeletal muscle cells, reportedly involving the small GTPase TC10, and independent of Rac1 or PI3K [215-217] . As in our skeletal muscle cells, N-WASP was implicated in actin remodeling in adipocytes, although use of transient over-expression of a dominant negative N-WASP mutant precludes the ability to conclude that N-WASP activation per se is responsible. Herein, we used WISK-mediated inhibition of N-WASP activation,

and this rapidly and thoroughly abolished insulin-induced cortical actin remodeling and decreased insulin-stimulated GLUT4 translocation in skeletal muscle cells. WISK inhibited these events during the insulin-stimulation period – no lengthy pre-treatment was required to halt these signaling events. This supports the concept that N-WASP activation, and not its synthesis or the synthesis of other proteins, underlies the PAK1-mediated cascade requiring N-WASP. Furthermore, that insulin-stimulated AKT phosphorylation remained unchanged in the presence of WISK, implicates N-WASP in a parallel pathway, leading to GLUT4 vesicle mobilization in skeletal muscle. An alternative to this would be that N-WASP acts downstream of AKT; this has not been demonstrated to date in any cell type to the best of our knowledge. This is consistent with data using inhibitors of PAK1 and Rac1, and the notion that a Rac1→PAK1→p41-ARC→N-WASP signaling pathway occurs independent of the established AKT signaling pathway in skeletal muscle cells.

In another new step in this cascade, insulin stimulation increased the association of N-WASP with Cortactin in mouse skeletal hindlimb muscle homogenates, and this was completely abolished in the skeletal muscle of global PAK1 $KO^{-/-}$ mice, indicating a role for PAK1 in regulating this interaction; this was fully supported in L6-GLUT4myc myoblasts. In non-muscle cell types, PAK1 has been shown to phosphorylate Cortactin at Thr21, and this phosphorylation is critical for the increased association of N-WASP with Cortactin to promote actin polymerization under stimulated conditions in Hep2b cells [124]. While the precise mechanism underlying insulin-stimulated N-WASP activation remains to be elucidated,

reports from other cell types suggest testable options. For example, N-WASP harbors an Cdc42/Rac interactive binding domain (CRIB), such that Rac1 could directly regulate N-WASP, over-stepping the need for PAK1 [218]. However, our data using IPA3 indicate a requirement for PAK1 in activating N-WASP in skeletal muscle cells. Alternatively, PAK1 and Rac1 could dually regulate the activation of Cortactin and N-WASP, respectively. Since Cortactin and N-WASP are known to synergistically activate ARP2/3 complex, via Cortactin binding to ARP3, and N-WASP binding to p41-ARC and ARP2 [219] this dual regulation hypothesis will need to be carefully examined.

This is the first identification of the polymerization arm of the actin remodeling cycle in skeletal muscle, involving p41-ARC-N-WASP--Cortactin interactions. However, this signaling cascade, overseeing the process of F-actin polymerization, must be managed with respect to the actin depolymerization signaling cascade, involving actin severing proteins such as Cofilin and SSH1, as these are also insulin regulated [121]. Indeed, the product of depolymerization is G actin monomers, and these monomers are the building blocks of F-actin filaments. Conceptually, it is by the compilation of these opposing arms of the actin remodeling cycle that the spatial and temporal rearrangement of cortical F-actin that creates tracks for the GLUT4 vesicles to move along to the PM. Future studies are warranted to discern the detailed mechanisms regarding insulin's ability to orchestrate the balance favoring actin polymerization, and actin *de*polymerization.

Altogether, our new findings place PAK1 as a central regulator of insulin sensitivity to maintain glucose homeostasis, and a reduction in PAK1 signaling/ abundance might be a potential risk factor for pre-diabetes susceptibility. Because PAK1 is such a central ‘hub’ for activities in actin remodeling in many cell types, identification of the primary pathways emanating from PAK1 in skeletal muscle will be important as a means to intervene beyond any PAK1 mutations or deficiencies. This will also circumvent any issues raised by retrospective samples associating PAK1 hyperactivation with various types of cancer [220]. Hence, efforts to restore PAK1 signaling leading to GLUT4 vesicle exocytosis in skeletal muscle carries potential to reinstate insulin sensitivity in pre-diabetic and Type 2 diabetic individuals.

CHAPTER 4

DISCUSSION

4.1. OVERVIEW

The objective of my dissertation was to determine the placement and function of PAK1 and its downstream signaling pathway(s) required for the maintenance of skeletal muscle insulin sensitivity. Toward this, the requirement of PAK1 signaling function in skeletal muscle glucose uptake was examined followed by determining the downstream signaling targets of PAK1 in regulating the cortical F-actin remodeling process, which is a pre-requisite for GLUT4 vesicle mobilization in skeletal muscle. Here, I will discuss findings from each chapter, alternative strategies and future studies required to further our understanding on the GLUT4 vesicle exocytosis process in skeletal muscle. Finally, the overall impact of the current findings in the control of whole body glucose homeostasis and strategies to prevent/ treat insulin resistance or T2D will be discussed.

4.1.1. Five major findings regarding PAK1 in skeletal muscle insulin sensitivity

PAK1 is the best-characterized member of the group I PAKs and is a widely-known regulator of cytoskeletal remodeling, cell survival, motility and proliferation. Previously, studies from our laboratory and others using whole body PAK1 KO mice revealed PAK1 to be a key critical required signaling protein in the process of insulin-stimulated GLUT4 translocation to the skeletal muscle cell surface. My studies corroborate these findings, and more importantly, further detail the function(s) of PAK1 in signaling downstream to previously unknown targets in skeletal muscle cells that lead to F-actin remodeling, eliciting glucose uptake into skeletal muscle. In addition to elucidating the signaling pathway emanating

immediately downstream of PI3K→Rac1 in skeletal muscle cells, five major discoveries from the current studies were made: 1) that PAK1 is activated by Rac1 in muscle cells, and its function is required for insulin-induced GLUT4 storage vesicle exocytosis and subsequent glucose uptake into skeletal muscle cells; 2) that PAK1 activation is required for the insulin-stimulated cortical actin remodeling in L6-GLUT4myc cells; 3) that PAK1 signals via a non-canonical LIMK-independent route to cofilin to regulate insulin-dependent F-actin remodeling in skeletal muscle cells; 4) that PAK1 regulates an atypical complex of actin-modifying proteins in skeletal muscle cells in response to insulin stimulation, including N-WASP, Cortactin and p41-ARC; and 5) that N-WASP functions in a PAK1-dependent manner to trigger insulin-stimulated F-actin remodeling in skeletal muscle cells.

4.1.2. PAK1 is involved upstream at the early step of initiating actin remodeling and triggering F-actin polymerization, in addition to depolymerization

My data established that although both PAK1 and PAK2 are present in L6-GLUT4myc myoblasts and skeletal muscle tissue, only PAK1 was implicated in insulin-induced GLUT4 mobilization to the cell surface. PAK2 knockdown was without effect upon insulin-stimulated GLUT4 translocation in skeletal muscle cells, and PAK3 was below the limit of detection both at mRNA as well as protein level in L6-GLUT4myc myoblasts and mature skeletal muscle tissue. Prior to my investigations PAK1 had been implicated in cofilin activation, and cofilin is involved in the *de*polymerization of F-actin. My research however showed that

PAK1 was actually involved upstream of this event, at the early step of initiating actin remodeling and triggering F-actin polymerization, via ARPC1, Cortactin and N-WASP complex assembly. In this manner, PAK1 initiates the start of the cycle of F-actin remodeling, first polymerizing F-actin into tracks, and then recycling via cofilin actions of depolymerization. Regarding cofilin regulation, in neuronal cells PAK1 was shown to activate its substrate LIMK, with LIMK then triggering cofilin phosphorylation [221]. Testing this in skeletal muscle cells, my research revealed that upon insulin stimulation PAK1 signals to activate cofilin (dephosphorylation) in an LIMK-independent pathway, suggesting the existence of an alternate pathway that leads to the dephosphorylation of cofilin. To date, there are only two known cofilin phosphatases, SSH and Chronophin proteins. Studies by Kligys *et al* revealed that Rac1 mediates the activation of cofilin by regulating SSH in keratinocytes via the action of 14-3-3, a phospho-serine/threonine binding protein [222]. With PAK1 being a serine/threonine kinase that has been previously shown to regulate the 14-3-3 binding to a GEF in non-muscle cell types [223], it is conceivable that PAK1 may activate SSH activity indirectly via the 14-3-3 protein to activate cofilin (dephosphorylation) in response to insulin in skeletal muscle cells.

4.1.3. PAK1 signals to p41-ARC to mediate ARP2/3 complex formation

On the other side of the actin remodeling cycle, the polymerization of actin was known to be regulated by the actin nucleating ARP2/3 complex in skeletal muscle cells [121]. My research extended this by identifying PAK1 as the upstream regulator of the ARP2/3 complex. A likely mechanism for this involves the PAK1-mediated phosphorylation of p41-ARC, an infrequently used regulatory subunit of the ARP2/3 complex. Prior to my work, ARPC1 was not identified nor linked with PAK1 in any cell type other that in MCF-7 breast cancer cells [122]. My studies also went on downstream to incorporate a function for N-WASP. While N-WASP was implicated in skeletal muscle signaling, it's activation and placement in the signaling cascade was unresolved. I was able to link this to PAK1 activity, using the pharmacological inhibitor of PAK1 (IPA3), which decreased the insulin-stimulated association of N-WASP with p41-ARC and actin, indicating the requirement for PAK1 signaling in regulating the interactions between these three proteins. Demonstration of pPAK1 utilizing p41-ARC as a substrate, and the subsequent phosphorylation of p41-ARC in an insulin-stimulated and PAK1-dependent manner remain to be confirmed in skeletal muscle cells. Moreover, PAK1 might also be involved in the regulation of microtubule dynamics to mediate GLUT4 vesicle translocation to the cell surface, by phosphorylating Ser65 and Ser128 of tubulin cofactor B (TCoB) [224]. This will need to be investigated, given that microtubules have been shown to be important for GLUT4 translocation and glucose uptake [204, 225]. Determining how PAK1

signaling interfaces with this process may uncover how GLUT4 vesicles transit across both networks to reach the cell surface.

4.1.4. Does PAK1 indirectly regulate the activity of N-WASP, via a Rac1-feed-forward cycle?

Another mechanism requires testing in skeletal muscle: that upon insulin stimulation, activated Rac1 binds to and activates PAK1, and PAK1 phosphorylates Rho-GDI, the guanine dissociation inhibitor isoform that associates with Rac1, initiating a positive feed-forward mechanism [204]. Plausibility for this arises from studies showing that Rac1 binds to a CRIB domain present within N-WASP to facilitate N-WASP activation, initiating actin remodeling [218]. In other cell types, PAK1 can act upstream of Rac1 [189]. However, there is no evidence at present for this mechanism in skeletal muscle, and hence additional studies are needed to evaluate the possibility of a positive feed forward activation of Rac1 by PAK1 in these cells.

4.2. FUTURE STUDIES

4.2.1. Inducible muscle specific PAK1 KO mice

Of the available skeletal muscle cell lines, the L6-GLUT4myc cell line is considered to be the most representative cell culture model of skeletal muscle function, enabling studies of the molecular mechanisms of skeletal muscle glucose metabolism in response to acute insulin signals. However, mature skeletal muscle differs from muscle cell culture models in their expression and reliance of various proteins involved in skeletal muscle glucose uptake. As such, studies using mouse models should be employed to validate the physiological

relevance of each protein and its network *in vivo*. While our lab and others have reported the whole body PAK1$^{-/-}$KO mice as having impaired glucose tolerance and insulin resistance, there is controversy as to which tissues, skeletal muscle, pancreatic α or β cells, gut or brain, is the dominant cause of the phenotype. Hence further studies using an inducible skeletal muscle-specific PAK1 KO$^{-/-}$ mouse model will serve the purpose of determining the role and requirement for PAK1 in the maintenance of glucose homeostasis via regulating skeletal muscle GLUT4 mobilization and subsequent glucose uptake. To determine if the PAK1 signaling network that my studies have unveiled is indeed conserved in the mature skeletal muscle glucose uptake process *in vivo,* the skeletal muscle from the inducible skm-PAK1 KO$^{-/-}$ mice should be analyzed for downstream signaling impairments of PAK1 (such as activation of Cofilin, ARP2/3 complex and N-WASP). In addition, since each of the three group I PAK isoforms possess a similar tertiary structure, function and mode of activation, it would be interesting to see if there is any compensation from the other two group I PAKs (PAK2 and PAK3) in the absence of PAK1. Subsequent to the publication of my studies implicating PAK1 in these processes, Skm-Rac1-KO$^{-/-}$ mice were evaluated and shown to exhibit an insulin-resistant phenotype with defects in PAK1 activation (phosphorylation). Furthermore, PAK1 signaling impairments in skeletal muscle were associated with an insulin resistant phenotype both in murine models, and in human tissues from T2D subjects. Hence, it would be important to determine whether Rac1 levels are modulated in the absence of PAK1, as well as the alternative pathways that have been observed to occur in non-muscle cell types,

which might mediate a compensatory response to the loss of PAK1 in skeletal muscle. If inducible skm-PAK1$^{-/-}$KO mice exhibit impaired glucose tolerance and insulin resistance, it would reiterate the importance of PAK1 in skeletal muscle glucose uptake and in the maintenance of whole body glucose homeostasis. However, it might also be possible that, the insulin resistant phenotype is instead due to the requirement for PAK1 in other tissues. In that case, if the inducible skm- PAK1$^{-/-}$KO mice were found to have normal GLUT4 exocytosis, PAK1's role in adipose-mediated glucose uptake in adipocytes should be evaluated, using adipocyte specific PAK1$^{-/-}$KO mice.

4.2.2. Muscle specific PAK1 overexpressing transgenic mice

PAK1 levels in T2D islets were ~80% reduced compared to levels present in their non-diabetic islets, implicating reduced PAK1 levels in diabetes. In contrast, PAK1 over-expression and hyper-activation has been associated with various types of cancers, with a large body of research focused upon inhibiting PAK1 as anti-cancer therapy [220]. These associations are correlative in nature however, with no side-by-side studies of a PAK1-overexpressing in vivo model compared with non- or hyper-activated PAK1 overexpressors. One report of cancer in a constitutively-active PAK1 overexpressing mouse model showed a more subtle phenotype than was expected or consistent with the correlative predictions derived from human tissue associations. Altogether, it can be presumed that anti-cancer drugs aimed at inhibiting PAK1 might render cancer patients susceptible to peripheral insulin resistance, metabolic dysregulation and the development of T2D. Hence, muscle-specific PAK1 overexpressing mice would be a valuable

tool to determine the consequences of over expressing PAK1 in skeletal muscle, as well as to verify whether increasing PAK1 levels in the muscle could serve as a means to improve the insulin sensitivity. Moreover, use of an inducible muscle-specific PAK1 overexpressing mouse model would permit the determination of whether PAK1 overexpression could protect against development of disease (induce the transgene prior to disease induction, e.g. via high-fat feeding, or crossing onto the db/db-BKS background) or could restore function to a diseased model (induce the transgene after the disease is developed).

4.2.3. Novel PAK1 interacting partners

PAK1 functions as a central node that mediates diverse functions of the cell by spatial-temporal regulation of its downstream effectors. PAK1 mediates the convergence of growth factor signaling and the adhesion signaling via activating the MAPK pathway by phosphorylating MEK1 on Ser298 [226]. Moreover, PAK1 phosphorylates Raf1 at Ser338, which translocates to mitochondria and binds to Bcl-2 (anti-apoptotic protein) and exerts its pro-survival or anti-apoptotic function [227]. PAK1 regulates cell cycle progression by phosphorylating MEK1 (Ser298) and Raf1 (Ser338) of the MAPK pathway. Moreover, phosphorylation of paxillin on S273 by PAK stimulates the localization of the signaling complex including adaptor protein G-protein coupled receptor kinase-interacting protein 1 (GIT1), the Rac GEF PAK interactive exchange factor (PIX) and the activated PAK to the leading edge to regulate adhesion and protrusion dynamics. Adding to this with my research, PAK1 signaling to initiate F-actin remodeling in skeletal muscle cells leads to insulin-stimulated GLUT4 translocation. This multitude of signals

from PAK1 signaling to its downstream targets to execute various cellular functions suggests that any variation of PAK1 activity from normal will be deleterious to the cell. Hence, identification of novel interacting partners of PAK1 which are specifically involved in the insulin-stimulated glucose uptake process, and not in the survival pathway, will provide us an opportunity to identify new potential therapeutic targets for T2D without affecting the normal survival/ anti-apoptotic pathways. Toward this, a proteomics approach combining mass spectrometric analysis and co-immunoprecipitation of PAK1 can be used to identify novel PAK1 interacting partners. Analyzing L6-GLUT4myc cells and mature skeletal muscle from unstimulated and insulin-stimulated conditions will provide us with PAK1 interacting partners that are specific to insulin stimulation which might include scaffolding as well as signaling partners. Identified novel PAK1 interacting partners in the insulin signaling pathway can be validated for their role and function in insulin-stimulated glucose transport in skeletal muscle cells by knockdown or overexpression studies, and confirmed for their requirement in mature skeletal muscle. Finally, selected downstream targets of PAK1 specifically involved in insulin-induced GLUT4 mobilization can be tested for druggability as the novel therapeutic targets for treatment and/or prevention of insulin resistance and T2D.

4.2.4. Correlation of PAK1 abundance and function with T2D susceptibility

Obese and T2D patients, and several mouse models of insulin resistance or diabetes, show deficiencies in gastrocnemius and soleus skeletal muscle [176].

PAK1 activation, cumulatively suggesting that dysregulated PAK1 signaling may be a significant contributor to the phenotype of peripheral insulin resistance, prediabetes and T2D. Therefore, diabetes-susceptible mouse models should be analyzed for PAK1 signaling impairments in the new proteins identified in the cascade in my studies, namely p41-ARC and Cortactin phosphorylations, and N-WASP activation in skeletal muscle. Further, Single nucleotide polymorphism (SNP) of PAK1 gene in T2D patients compared to that of healthy individuals should be assessed to establish a correlation between PAK1 and T2D. Overall, these studies will aid in predicting the threshold of PAK1 protein levels or function in skeletal muscle, required to maintain normal insulin action and ways to restore PAK1 abundance/function in these tissues can later be exploited to maintain whole body glucose homeostasis.

4.3. CONCLUSIONS

Overall my above studies revealed that PAK1 functions downstream of Rac1 in an LIMK-independent manner in the process of insulin-stimulated glucose uptake in skeletal muscle cells. Ablation of PAK1 function resulted in abrogation of F-actin remodeling dependent GLUT4 mobilization to the skeletal muscle cell surface in response to insulin. Interestingly, PAK1 regulates cofilin activity to induce insulin-dependent F-actin remodeling in a manner distinct from the other cell types, however the mechanism is yet to be determined. Moreover, PAK1's role in the regulation of N-WASP-p41-ARC-actin complex to initiate cortical actin remodeling revealed a novel insulin signaling pathway that facilitates glucose uptake into skeletal muscle. Altogether, this study is significant because it is the

essential first step towards the development of pharmacologic strategies that will facilitate preservation of PAK1 function in skeletal muscle insulin action which are crucial to maintain glucose homeostasis.

CHAPTER 5

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Curriculum Vitae

Ragadeepthi Tunduguru

EDUCATION

PhD 2011-2016
Department of Biochemistry and Molecular Biology
Indiana University, Indianapolis IN, USA

M.Sc 2003-2005
Department of Biochemistry.
Bangalore University, Karanataka, India

RESEARCH EXPERIENCE

Industrial experience: AstraZeneca India Pvt Ltd

- As an assistant research scientist at AstraZeneca India Pvt Ltd, I was involved in developing and performing biochemical, microbial and radioactive assays to determine the mode of action of candidate drugs in microorganisms including E.coli and M.bovis. I was also involved in the assessment of early sterilizing potential of new anti-tuberculosis molecules using immunological markers as surrogates for bacterial clearance and to do away with the time consuming CFU counting.

Doctoral research: Indiana University School of Medicine, Indianapolis, IN
Advisor: Dr. Debbie C Thurmond
Project: Role of PAK1 signaling in skeletal muscle insulin sensitivity

- Currently, investigating the function and placement of PAK1 and actin regulatory proteins in skeletal muscle glucose transport using both in vitro (variety of fixed cell and live cell confocal immunofluorescent techniques, analysis of proteins and protein-protein interactions by immunoprecipitation technique), ex vivo (GLUT4 translocation and radioactive glucose uptake in skeletal muscle tissue) and in vivo (phenotypic and metabolic analyses of mouse models) by using skeletal muscle cell line and skeletal muscle specific PAK1 KO mouse model.

FUNDING, AWARDS and FELLOWSHIPS

American Heart Association (AHA)
Predoctoral Jan 2015 - Dec 2016

Second place on Research talk, Biochemistry Research Day 2014

ASBMB 2014 Graduate Student Travel Award 2014

IUSM Graduate Student Travel Award 2013

Promoted to Assistant Research Scientist -2 at AstraZeneca

India Private Ltd 2009

Award of Excellence at AstraZeneca, India 2008

Best Team award for the year 2007 in AstraZeneca India Private Ltd 2007

Promoted to Assistant Research Scientist -1 at AstraZeneca
India Private Ltd 2006

Recipient of B. V. Ramananmma Memorial award for proficiency in
Organic Chemistry 2003

Collector's endowment for Proficiency in Biochemistry 2003

PUBLICATIONS

1. Ahn M, Yoder S M, Wang Z, Oh E, Ramalingam L, Tunduguru R, Thurmond D C. (2016) The p21-Activated Kinase (PAK1) is involved in diet-induced beta cell mass expansion and survival in mice and human islets. Diabetologia. 2016 Oct;59(10):2145-55. PMID: 27394663

2. Veluthakal R , Tunduguru R, Arora D K, Sidarala V, Vlaar C P, Thurmond D C, Kowluru A. (2015) Vav2, a guanine nucleotide exchange factor for Ras-related C3 botulinum toxin substrate 1 (Rac1), regulates glucose-stimulated insulin secretion in pancreatic β-cells. Diabetologia. 2015 Nov;58(11):2573-81. PMID: 26224100

3. Ravishankar S, Ambady A, Ramu H, Mudugal N V, Tunduguru R, Sambandamurthy V K, Ramanna S. (2015) An IPTG inducible conditional expression system for mycobacteria. Plos One. 2015 Aug 6;10(8):e0134562. PMID: 26247874

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5. Reddy BK, Landge S, Ravishankar S, Patil V, Shinde V, Tantry S, Kale M, Raichurkar A, Menasinakai S, Mudugal NV, Ambady A, Ghosh A, Tunduguru R, Kaur P, Singh R, Kumar N, Bharath S, Sundaram A, Bhat J, Sambandamurthy VK, Björkelid C, Jones TA, Das K, Bandodkar B, Malolanarasimhan K, Mukherjee K, Ramachandran V. (2014).

Assessment of Mycobacterium tuberculosisPantothenate kinase vulnerability through target knock down and mechanistically diverse inhibitor.Antimicrobial Agents and Chemotherapy. 2014 Jun;58(6):3312-26. PMID: 24687493.

6. Ramachandran, V., Singh, R., Yang, X., Tunduguru, R., Mohapatra, S., Khandelwal, S., Patel, S., and Datta, S. (2013). Genetic and chemical knockdown: a complementary strategy for evaluating an anti-infective target. Advances and applications in bioinformatics and chemistry. 2014 Jun;58(6):3312-26. PMID: 23413046.

7. Balasubramanian, V., Solapure, S., Iyer, H., Ghosh, A., Sharma, S., Kaur, P., Tunduguru R Subbulakshmi, V., Ramya, V., Ramachandran, V., et al. (2013). Bactericidal activity and mechanism of action of AZD5847: a novel oxazolidinone for the treatment of tuberculosis. Antimicrobial agents and chemotherapy. 2014;58(1):495-502. PMID: 24189255.

PRESENTED ABSTRACTS

1. Tunduguru R, Zhang J, Elmendorf J.S, Thurmond D C. N-WASP-Cortactin signaling promotes skeletal muscle GLUT4 translocation. Presentation at the American Diabetes Association meeting in New Orleans, LA June 10-14, 2016.

2. Tunduguru R, Chiu T T, Klip A, Thurmond D C. Insulin-stimulated PAK1 activation is required for glucose uptake in skeletal muscle. Presentation at the Experimental Biology meeting in San Diego, CA April 26-30, 2014.

3. Balakrishnan A, Tunduguru R, David S, Barde S, Muruguppan R, Ramachandran V. Amino acid pool extraction and analysis from M.bovis. ICGEB (International Centre for Genetic Engineering and Biotechnology) New Delhi, December 11-13, 2006.